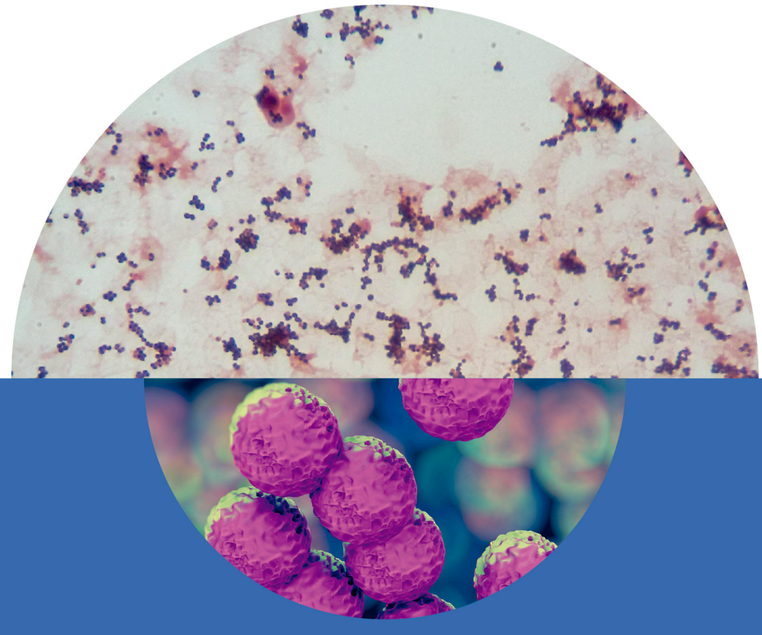
Methicillin-resistant *Staphylococcus aureus*: evolution of endemic clones and emergence of community clones in the hospital environment



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Mariana Adrião Camoez **Methicillin-resistant *Staphylococcus aureus*:** evolution of endemic clones and emergence of community clones in the hospital environment **PhD Thesis 2015**

Bellvitge Hospital
IDIBELL Institut d'Investigació Biomèdica de Bellvitge
reipi Red Española de Investigación en Patología Infecciosa

UNIVERSITAT DE BARCELONA

Programa de Doctorat en Biomedicina

Línea: Metabolisme, Senyalització Metabòlica i Patologies Associades

Facultat de Medicina - Campus de Bellvitge

Departament de Patologia i Terapèutica Experimental

"Methicillin-resistant *Staphylococcus aureus*: evolution of endemic clones and emergence of community clones in the hospital environment"

Dissertation presented to obtain the Ph.D degree in Biomedicine

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Barcelona, November 2015

To the memory of my grandfather Raúl Adrião

This thesis was funded by two grants from Fondo de Investigación Sanitaria (grants number PI 07/0944 and PI 10/01783).

The PhD student was supported by Ministerio de Economía y Competitividad, Instituto de Salud Carlos III - co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008 and RD12/0015).

After six years of intensive work and personal efforts, I present my PhD thesis. Working on the Ph.D. has been a wonderful and often overwhelming experience. It has been hard to deal with the real learning experience, how to write papers and proposals, give talks, work in group, collaborate with residents and Master's students, stay up until the birds start singing, and stay focus... In any case, I am grateful to many people for making the time working on my Ph.D. an unforgettable experience.

En primer lugar, quiero agradecer a mi directora de tesis, la Dra. M. Ángeles Domínguez por haber confiado en mí y permitirme llevar a cabo el proyecto de tesis doctoral en su laboratorio, creer en mi capacidad de trabajo y enseñarme el fascinante mundo del *Staphylococcus aureus.* Además de un agradecimiento profesional, también quiero agradecer a M. Ángeles Domínguez su amistad que me ayudó a superar algunos momentos difíciles de mi vida personal, sin ella todo hubiera sido más difícil en los últimos 6 años en Barcelona.

A la Dra. Fina Liñares por contagiarme el interés por los estudios de actividad de la vancomicina/daptomicina e interesarse por mi situación profesional, a pesar de no tener una responsabilidad directa conmigo. Un ejemplo a seguir como profesional y como persona.

Otro agradecimiento muy especial lo quiero dedicar a la Dra. Fé Tubau por su infinita disponibilidad en enseñarme muchísimo sobre los mecanismos de resistencia en *Staphylococcus aureus* y en *Pseudomonas aeruginosa*, en ayudarme con los estudios de sensibilidad antibiótica, incluso en sus atareados días de guardia. Su sonrisa, sus chocolatinas y sus palabras amables cuando más lo necesitaba, me ayudaron a que las horas extras en laboratorio fuesen más placenteras.

También quiero agradecer al Dr. Jordi Niubó por su disponibilidad en ayudarme siempre que lo necesité, sobre todo al principio cuando generosamente (con algunos deliciosos croissants de soborno) me prestaba sus termocicladores para que pudiera avanzar en mi proyecto de tesis.

Muchas gracias también al Dr. Rogelio Martín, jefe del Servicio de Microbiologia del Hospital Universitari de Bellvitge (HUB) cuando llegué por primera vez. Por su buen humor y por la amabilidad y disponibilidad en ayudarme siempre que he necesitado.

Muchas gracias a todo el personal técnico del Servicio de Microbiología. En especial quería agradecer a tres personas del personal técnico del laboratorio: Mercedes, Joel y Encarna. A Mercedes y a Joel por todos los años de poyata, entreayuda, charlas y risas que hemos compartido y que jamás olvidaré. À Encarna por el cariño y apoyo que me demostró desde el principio de mi doctorado hasta al día de hoy.

Gracias a los antiguos y nuevos residentes de Microbiología del HUB por la amabilidad y disponibilidad para ayudarme en mis proyectos, incluso en algún día de guardia. Las más veteranas Anabel y Zay, dos apoyos incondicionales al principio de mi tesis, tanto en el plano laboral como en el amistoso fuera del hospital, que aún continúa a día de hoy. Chema y Mª José, que han terminado su residencia recientemente. Ha sido muy gratificante conocerlos, tanto en laboratorio como fuera de él. Y finalmente Dàmaris, una residente llena de alegría, a la que le gusta tanto como a mí, la Microbiología y la Investigación. Muchas gracias por las muchas horas de charlas interesantes y por las risas que nos hemos echado.

También quiero agradecer a los demás facultativos del servicio que siempre estuvieron disponibles para ayudarme en el laboratorio. Por orden alfabético: Dr. Fernando Alcaide, Dra. Carmen Ardanuy, Dra. Fina Ayats, Dr. Jordi Càmara, Dra. Laura Calatayud y Dra. Dolors García-Somoza

A la Red Española de Investigación de Patologías Infecciosas (REIPI) por otorgarme un contrato de investigación, sin lo cual todo esto no hubiera sido posible. Al Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), por gestionarme el contrato predoctoral y viajes a congresos, además de cederme el despacho, donde he podido con más tranquilidad analizar los datos y escribir esta tesis doctoral. A la Universitat de Barcelona (UB) y al programa de Doctorado de Biomedicina, gracias. Gracias a la Dra. Teresa Vinuesa, al

Profesor Miquel Viñas y a la Comisión de Doctorado por haber supervisado mi proyecto.

Otra ayuda fundamental para la realización de esta tesis, ha sido la colaboración con los doctores del Servicio de Infecciosas del HUB, el Dr. Miquel Pujol y el Dr. Oriol Gasch. Aprendí muchísimo con sus estudios clínicos, sobre todo con el proyecto FIS múlticéntrico, en el cual tuve la oportunidad de colaborar activamente, y del cual han surgido varios resultados microbiológicos presentados en esta tesis y varias publicaciones en las cuales soy 2ª firmante. Gracias por su profesionalidad, por su disponibilidad para comentar la historia clínica de los pacientes y valorar los resultados microbiológicos, porque detrás de un aislamiento de *Staphylococcus aureus*, siempre hay un paciente.

Al Dr. Jordi Vila, al Dr. Ignasi Roca y al Dr. Mario Ferrer-Navarro del Servicio de Microbiología del Hospital Clinic por la importante colaboración en el trabajo sobre la discriminación automatizada de los diferentes linajes de *Staphylococcus aureus* resistente a meticilina por MALDI-TOF y en su posterior publicación.

To Professor Hermínia de Lencastre, Head of the Laboratory of Genetics at the Instituto de Tecnologia Química e Biológica (ITQB), Oeiras, Portugal, and Dr. Teresa Conceiçao and collaborators, especially Dr. Maria Miragaia for receiving me, for your guidance among MRSA, and particularly for your friendly atmosphere.

Como no, agradecer a mis compañeros de investigación que han estado a mi lado y que han contribuido directamente o indirectamente para el desarrollo de esta tesis. Por orden alfabético: Arnau, Carmen, Dora, Dani, Javi, Jennifer, Josep, Meritxell, Montse, Núria, Raquel, Raúl y Sara. Me gustaría agradecer especialmente a algunos de ellos. A Carmen, mi mejor compañera de viaje a congresos. Gracias por tu consejos y también porque en los últimos tiempos te convertiste en un punto de apoyo importante en la elaboración de esta tesis. A Dora, una compañera de trabajo y una amiga fuera del laboratorio desde que llegué al HUB. Todo empezó con una magdalena en el bar del HUB, y hoy no soy capaz de contar mi vida en Barcelona sin hablar de todo que he vivido contigo y lo que me has ayudado. Ah y por supuesto tu hijo Sebastião, a quién

quiero como si fuera de mi familia. A Javi por tus conversaciones interesantes a la hora de la comida y por tu energía y optimismo en el trabajo. A Meritxell, mi amiga desde el Máster de Microbiología Avanzada en la UB. Gracias por todo el apoyo que me has dado todos estos años tanto a nivel profesional como a nivel personal. Sé que siempre podré contar contigo. A Raúl (el cocinillas del laboratorio), a pesar de que ya no estés en el HUB, gracias por tu disponibilidad en ayudarme siempre que te he necesitado. Lo único negativo que hay que decir de ti, es que aún espero mi “coulant de chocolate”. A Sara, mi compañera de Sushi. Agradecerte todo el apoyo que me has dado en los últimos años de mi tesis. Y finalmente agradecer a Raquel, una amiga con quién he compartido muchas anécdotas, tanto en el laboratorio como fuera de él. Siempre pude contar contigo cuando te necesitaba. Gracias por los numerosos momentos divertidos que pasé contigo, ¡pero sobre todo por los momentos de complicidad y de ayuda mutua que hemos vivido!

Gracias a mis compañeras del despacho por aguantarme a diario y transformar los últimos meses de más *stress* en días más placenteros y tranquilos. Por orden alfabético: Antonella, Isa y Lorena.

Às minhas amigas portuguesas, Ana Duarte (uma grande amiga de infância), Diana Barbosa (uma lutadora e uma amiga muito especial que conheci quando trabalhei no “Consejo Superior de Investigación Científica, CSIC”), Diana Espadinha (a melhor surpresa do meu estágio no ITQB), Ana Silva (uma grande amiga da universidade) e Rita Lino (a melhor amiga e a mãe do meu querido afilhado Duarte), pela vossa eterna amizade e por estarem sempre a meu lado, um grande obrigado por tudo!

Ao Pedro, queria agradecer por ter estado ao meu lado, sobretudo nestes últimos meses da tese, por ter tido a paciência de me ouvir e de aturar as minhas crises de *stress*, pelo seu amor e companhia ao longo destes anos (que pensávamos ser quatro!) e que não foram fáceis para os dois.

Por último, agradecer à minha família, em especial aos meus pais, Adelaide e Manuel, aos meus irmãos Ricardo e Diogo, à minha avó Alice e à minha tia Irene pelo apoio incondicional que me deram nesta longa caminhada.

Um obrigado muito especial à minha mãe pela paciência, amor e força que me transmitiu durante todos estes anos (horas e horas de telefone!).

Esta tese é dedicada à memória do meu querido avô Raúl, um homem humilde que foi um exemplo de trabalho e de dedicação aos seus netos, para quem sonhou um percurso académico, um curso superior que a vida não lhe deu a oportunidade de realizar. O amor que me dedicou a par dos princípios e valores que me legou foram inspiradores e encorajadores para fazer este caminho. Onde ele estiver, tenho a certeza que estará orgulhoso de mim.

Abstract

Infections by methicillin-resistant *Staphylococcus aureus* (MRSA) are still an important cause of morbidity and mortality worldwide. Health-care associated infections, especially bacteremia, often represent a clinical challenge because of the severity of the infection, the associated high mortality (close to 30% in some series) and the difficulty to initiate a correct empirical (o guided) antibiotic therapy.

MRSA has spread throughout Hospital Universitari de Bellvitge (HUB) for more than 20 years and, in spite of preventive measures, the percentage of methicillin resistance among *S. aureus* is currently close to 24%. This figure is similar to that found in many other hospitals around Spain. The epidemiology of MRSA infection has been changing over the years with the evolution of endemic clones and the emergence of new genetic lineages of MRSA with distinct virulence attributes and with the ability to spread within the community. The identification of new reservoirs and the knowledge of the MRSA genetic content will help on one hand, to better understand the pathogenesis of the disease and to facilitate the adequate therapy; and, on the other hand, to limit the spread of epidemic lineages.

The aim of the work presented in this Thesis was to provide an integrated analysis of the genetic background of successive MRSA populations causing infection in the HUB since the beginning of the endemia (1990-2014), linked to the phenotypic expression of antibiotic resistance. In addition, we were the reference Microbiology Laboratory for a multicentre study that allowed us to have access to MRSA bacteremic isolates from 23 different hospitals in Spain obtained in 2008-2009.

The main *objectives* of this work were:

1. *To analyse the evolution of methicillin resistance among S. aureus strains, isolated from 1990 to 2014 in the HUB, and to study MRSA's antimicrobial susceptibility to old and new antistaphyloccocal therapeutic agents*.

To address this objective we developed different studies:

- Analysis of the evolution of MRSA's antimicrobial susceptibility to conventional antibiotics.
- Study of *in vitro* activity of daptomycin and vancomycin against MRSA blood isolates.
- Impact of accessory gene regulator (*agr*) and presence of *rpo*B mutations on the phenotypic expression of vancomycin susceptibility
- Study of transmissible resistance to linezolid mediated by *cfr* gene.

2. *To describe the molecular epidemiology of emergent and endemic MRSA clones, isolated in HUB from 1990 to 2014, using genotypic and proteomic-based methods.*

To undertake this goal we applied standard typing methods (pulsed-field gel electrophoresis, SCC*mec*, *agr* and *spa* typing, and multilocus sequence typing), to describe the endemic and the new MRSA lineages identified. We developed an automated strategy for typing MRSA applying the MALDI-TOF system. In addition, we determine the presence of genes codifying Panton-Valentine leukocidin and the arginine catabolic mobile element (ACME).

To end the molecular characterization of MRSA isolates, we explore the presence of a panel of virulence and resistance genes.

3. *To determine whether the changes detected in the MRSA endemic population in HUB were similar in other clinical settings.*

To address this objective we applied the same methodologies from objectives 1 and 2 to a selection of 626 MRSA bacteremic isolates from 23 Spanish hospitals.

The results of the studies addressed in this Thesis showed that, on average, 20% of all *S. aureus* isolated in Hospital Universitari de Bellvitge (HUB) from 1990 to 2014, were methicillin resistant. Over the last 10 years, methicillin resistance percentage was around 24%. This figure is similar to other hospitals in Spain (25%).

The dominant lineage in HUB for the first decade (the multiresistant Iberian clone, ST247-SCC*mec*I-*agrI*) was considered extinct. Currently, Clonal Complex 5, related to the Pediatric clone (ST5-SCC*mec*IV-*agrII*), was the dominant lineage in the HUB, as it was in other Spanish hospitals. In the HUB, clone ST8-SCC*mec*IV-*agr*I has become the second most frequent lineage in 2014. Antibiotic resistance determinants as well as genetic determinants of virulence properties were specific of particular clones.

The presence of community-acquired MRSA lineages was detected in both MRSA collections (MRSA isolated from HUB and MRSA from different Spanish hospitals), being the most important clone USA300 (with positive and negative ACME) and the livestock related clone MRSA-ST398. However, the number of isolates belonging to these clones was scarce and in most cases corresponded to hospital-onset infections, caused by bacteria acquired in the community. No

significant nosocomial transmission of community-acquired clones was detected.

Vancomycin tolerance was only detected among isolates belonging to clone ST247-SCC*mec*I-*agrI* (Iberian clone). In addition, we could not detect any influence on vancomycin susceptibility expression of *agr* polymorphism or *rpoB* mutations.

Resumen

Las infecciones por *Staphylococcus aureus* resistente a la meticilina (SARM) siguen siendo una causa importante de morbilidad y mortalidad en todo el mundo. Las infecciones asociadas al sistema sanitario, especialmente las bacteriemias, suelen representar un desafío clínico debido a la severidad de la infección, la mortalidad elevada (cerca del 30%) y la dificultad para iniciar el tratamiento antibiótico empírico adecuado.

SARM se ha diseminado en el Hospital Universitari de Bellvitge (HUB) durante más de 20 años y a pesar de la aplicación de medidas preventivas, actualmente el porcentaje de resistencia a meticilina entre los aislamientos de SARM está cerca al 24%. Esta situación es similar a muchos otros hospitales españoles. La epidemiologia de la infección por SARM ha cambiado al largo de los años con la evolución de los clones endémicos y la aparición de nuevos linajes de SARM con distintos perfiles de virulencia y con la capacidad de diseminarse en la comunidad. La identificación de nuevos reservorios y el conocimiento de la patogénesis de la enfermedad facilitarán la aplicación de la terapia adecuada y por otro lado, pueden contribuir a limitar la diseminación de linajes epidémicos. El objetivo del trabajo presentado en esta tesis fue realizar un análisis integrado del perfil genético de las sucesivas poblaciones de SARM que han causado infección en el HUB desde el principio de la endemia (1990-2014), y un estudio de la expresión fenotípica de la resistencia antibiótica. En, este trabajo, se incluye también los resultados de un estudio multicéntrico en el que el Servicio de Microbiología del HUB actuó como centro de referencia, para el estudio de aislamientos procedentes de pacientes con bacteriemia por SARM ingresados en 23 hospitales españoles aislados durante el período 2008-2009.

Los principales objetivos de este trabajo fueron:

1. *Analizar la evolución de la resistencia a meticilina en las cepas de S. aureus aisladas desde 1990 a 2014 en el HUB, y estudiar la sensibilidad antibiótica a los antiguos y nuevos agentes terapéuticos antiestafilocócicos.*

Para completar este objetivo se desarrolló los diferentes estudios:

- Análisis de la evolución de la sensibilidad antimicrobiana de los aislamientos de SARM a los antibióticos de uso clínico común.
- Estudio de la actividad *in vitro* de la daptomicina y vancomicina frente a los aislamientos bacteriémicos de SARM.
- Influencia del tipo de *agr* y de distintas mutaciones en el gene *rpoB,* en la expresión fenotípica de la sensibilidad a vancomicina.
- Estudio de la resistencia transmisible al linezolid mediante el gen *cfr*.

2. *Describir la epidemiologia molecular de los clones endémicos y emergentes de SARM, aislados en el HUB desde 1990 hasta 2014, utilizando técnicas genotípicas o proteómicas.*

Para llevar a cabo este objetivo se utilizaron métodos de tipificación genética de referencia (electroforesis en campo pulsado, tipaje por la técnica “multilocus sequence typing”, tipaje del SCC*mec*, del *agr* y de la proteína *spa*), para describir las familias del SARM endémico y los nuevos linajes. También se desarrolló una estrategia de tipaje del SARM aplicando el sistema MALDI-TOF. Además, se determinó la presencia de los genes que codifican la leucocidina de Panton-Valentine (LPV) y el elemento genético móvil que codifica para el catabolismo de la arginina (ACME). Finalmente, se completó la caracterización

molecular de los aislamientos de SARM mediante un amplio estudio de sus genes de resistencia antibiótica y virulencia.

3. *Determinar si los cambios detectados en la población endémica de SARM en el HUB fueron similares en otros hospitales.*

Para llevar a cabo este objetivo, se utilizó la misma metodología que en los objetivos 1 y 2, en una colección de 626 aislados bacteriémicos procedentes de 23 hospitales españoles.

Los resultados de los estudios presentados en esta tesis muestran que la resistencia media a meticilina en *S. aureus* alcanzó un valor del 20% en el HUB en el período 1990-2014. Durante los últimos 10 años, el porcentaje de resistencia a meticilina fue próximo al 24%. Esta situación fue similar en los demás hospitales (25%). El linaje dominante en el HUB durante la primera década del estudio (el clon multiresistente – clon Ibérico, ST247-SCC*mec*I-*agr*I), desapareció posteriormente. Actualmente el complejo clonal 5, relacionado con el clon Pediátrico (ST5-SCC*mec*IV-*agr*III) es el linaje dominante en el HUB, así como en los demás hospitales en España. En el HUB, el clon ST8-SCC*mec*IV-*agr*I se ha convertido en el segundo clon más frecuente en 2014. Los determinantes de resistencia antibiótica así como los determinantes genéticos de virulencia fueron específicos para cada clon. La presencia de linajes de origen comunitaria se detectó en ambas colecciones de SARM (SARM aislado en el HUB y SARM aislado de diferentes hospitales españoles), siendo los más importantes los clones USA300 (ACME positivo y negativo) y el clon SARM asociado al ganado - SARM-ST398. Sin embargo, el número de aislados que pertenecieron a estos clones fue bajo y en la mayoría

de los casos se asociaron a infecciones que debutaron en el hospital, causadas por bacterias adquiridas en la comunidad. No se detectó transmisión nosocomial de los clones asociados a la comunidad. La tolerancia a la vancomicina solamente se detectó en aislamientos que pertenecían al clon ST247-SCC*mec*I-*agr*I (clon Ibérico). No se detectó ninguna influencia del tipo del polimorfismo del *agr*, ni de las mutaciones del gene *rpoB* en la expresión de la sensibilidad a la vancomicina.

Abbreviations

Abbreviations

A

ACME	arginine catabolic mobile element
AFLP	amplified fragment length polymorphism
agr	accessory gene regulator
AIP	autoinducing peptide
arcC	carbamate kinase
arlR/*arlS*	autolysis-related locus sensor
aroE	shikimate dehydrogenase

B

Bac	bacteriocins
bbp	bone sialoprotein-binding protein
Bsa	bacteriocin of *S. aureus*
BURP	based upon repeat pattern

C

C; CLO	chloramphenicol
CAMHB	cation adjusted Mueller Hinton broth
CAMPs	cationic antimicrobial peptide
CA-MRSA	community-associated MRSA
cap	capsular polysaccharide
CC; CLI	clindamycin
CC	clonal complex; genetic backgrounds
CCDP-DG	CDP-diacylglycerol

ccr	cassette chromosome recombinases
CDC	Center for Disease Control and Prevention
CF	cystic fibrosis
clfA	clumping factor A
clfB	clumping factor B
cfr	chloramphenicol-florfenicol resistance
CFU	colony-forming unit
CHIPS	chemotaxis inhibitory protein of staphylococci
CIP	ciprofloxacin
CLSI	Clinical Laboratory Standard Institute
cna	collagen-binding protein
Coa	coagulase
CoNS	coagulase-negative staphylococci
csl2	cardiolipin synthetase
COS	columbia agar sheep blood
CV	core variable

D

DA; DAP	daptomycin
DLV	double-locus variant
DNA	deoxyribonucleic acid

E

E; ERY	Erythromycin
Eap	extracellular adherence protein

EARS-NET	European Antimicrobial Resistance Surveillance Network
ebh	fibronectin-binding protein
EbpS	elastin binding protein S
eBURST	electronic based upon related sequence types
ETA	exfoliative toxin A
EUCAST	European Committee on Antimicrobial Susceptibility Testing

F

FDA	Food and Drug Administration
FnBPA/B	fibronectin-binding protein A and B
FOX	cefoxitin

G

GA	genetic algorithm
glpF	glycerol kinase
GM; GEN	gentamicin
gmk	guanylate kinase
goeBURST	global optimal eBURST

H

HA-MRSA	hospital-associated MRSA
hetero-VISA	heterogeneous vancomycin intermediate *S. aureus*
Hla	alpha-hemolysin
hlb	β-hemolysin
HUB	Hospital Universitari de Bellvitge

I

i	Intermediate resistant
icaACD	intercellular adhesion biofilm genes A, C, and D
IEC	immune evasion cluster
isdA	Iron-regulated surface determinant
IWG-SCC	International Working Group on the Staphylococcal Cassette Chromosome elements

J

J	joining

L

LA-MRSA	livestock-associated MRSA
LPG	lysylphosphatidylglycerol
LZD	linezolid

M

MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
MBC	minimal bactericidal concentration
MDR	multidrug resistant
MGE	mobile genetic element
mgr	multiple gene regulator
MH2	mueller-hinton II
-MHC	major histocompatibility complex
MIC	minimum inhibitory concentration

MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MLVA	multiple locus variable number of tandem repeat analysis
MRSA	methicillin-resistant *S. aureus*
MS	mass spectrometry
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
MSP	main spectrum
MSSA	methicillin-susceptible *S. aureus*
m/z	mass to charge

N

NN	tobramycin
NT*Sma*I	Inability to be typed by *Sma*I restriction

O

OD	optical density
ORF	open reading frame
OX	oxacillin

P

P	penicillin
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PG	phosphotidylglycerol

	phenicols, lincosamides, pleuromutilines and streptogramin
PhLOPS	A
PIA	polysaccharide intercellular adhesion
PMNs	polymorphonuclear leukocytes
ppm	parts per million
psgA	phosphatidyglycerol synthase
PSMs	phenol-soluble modulins
pta	phosphate acetyltransferase
PVL	Panton-Valentine leukocidin

Q

QC	quickclassifier
QS	quorum sensing

R

r	resistant
RA	rifampicin
RAPD	random amplified polymorphic DNA
REIPI	Spanish Network for Research in Infectious Diseases
rot	repressor of toxins
RP	antibiotic resistance patterns

S

s	susceptible
sae	staphylococcal accessory element
SAgs	superantigens

SAK	staphylokinase
SaPI	staphylococcal pathogenicity islands
sar	staphylococcal accessory regulator
sasG	*S. aureus* surface protein G
SCC*mec*	staphylococcal cassette chromosome *mec*
SCIN	staphylococcal complement inhibitor
SCVs	small colony variants
sdrC/D/E	fibrinogen-binding proteins C, D and E
SE	staphylococcal enterotoxins
sigB	staphylococcal alternative sigma factor B
SLV	single-locus variant
SNN	supervised neural network
SNPs	single-nucleotide polymorphisms
spa	*S. aureus* protein A
*spa*CC	*spa* clonal complexes
SSR	short sequence repeats
SSTIs	skin and soft tissue infections
STs	sequence types
SXT	trimethoprim-sulphamethoxazole
SYN	quinupristin-dalfopristin

T

TE; TET	tetracycline
TEC	teicoplanin
Tet-R	tetracycline resistance

TLV	triple-locus variant
TOB	tobramycin
tpi	triosephosphate isomerase
TSA	tryptic soy agar
TSB	tryptic soy broth
TSS	toxic shock syndrome
TSST-1	toxic shock sindrome toxin-1

U

UK	United Kingdom

V

VA; VAN	vancomycin
VISA	vancomycim intermediate *S. aureus*
vWbp	von Willebrand factor binding protein

W

WTA	wall teichoic acids

Y

yqiL	acetyl coenzyme A acetyltransferase

Table of contents

Chapter I

Introduction

1. *Staphylococcus aureus*

Staphylococcus aureus is a highly successful opportunistic pathogen that is able to colonize humans and animals and can cause a wide variety of diseases [1].

1.1 Microbiologic characteristics

In 1882, Alexander Ogston named *Staphylococcus* to describe micrococci that produced inflammation and suppuration [2]. However, it was Friedrich Julius Rosenbach in 1884, who introduced the first taxonomic description of *Staphylococcus aureus;* the genus name was derived from the Greek *"staphylé"* (for bunch of grapes) and the species epithet "*aureus*" from the Latin word for gold, suggested by the clear tendency of these bacteria to grow in clusters and the golden appearance of the colonies, often with hemolysis, when grown on blood agar plates [3] (see Figure 1).

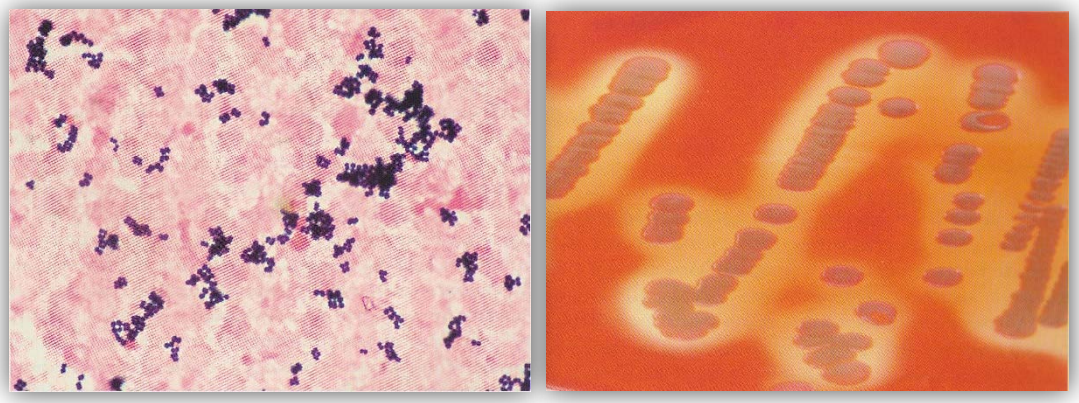


Figure 1: (A) Gram stain of *S. aureus* cells which typically occurs in grape-cluster berry; (B) Yellow colonies and β-hemolytic of *S. aureus* on a blood agar plate (taken from [4]).

The genus *Staphylococcus* is currently composed of 51 species and 27 subspecies [5]. Only a few of these species are pathogenic in the absence of

immunosuppression or implanted foreign material. The most important etiologic agents for infection in humans include *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. schleferi*, *S. warneri* and *S. saprophyticus*. Despite *S. aureus* being the specie more pathogenic and virulent found in humans, it can exist as resident or a transient member of the normal flora [1].

Staphylococci sharing the following microbiological traits were placed in the family *Micrococcaceae*: gram-positive cocci (0.5 to 1.5 µm in diameter) with diverse arrangements (singly and in pairs, tetrads, short chains, and irregular grape-like clusters), with unique cell wall peptidoglycan with pentaglycine interpeptide bridges, which is the target of the lysostaphin activity, non-motile bacteria, non-spore forming, facultative anaerobes, glucose fermenting, usually catalase positive, oxidase negative, often unencapsulated or with a limited capsule, and most of the species being able to grow at 1.7M of NaCl (salt-tolerant) [1], [3], [6]–[9].

In general, *Staphylococcus aureus* could be identified by the production of the enzyme coagulase, which is a cell surface-associated fibrinogen-binding protein, secreted during bacterial growth that clots the human plasma. This characteristic allows to discriminate between coagulase positive (i.e., *S. aureus*) and coagulase-negative staphylococci (CoNS) [1]. Only two animal-specific coagulase positive species have been described, *Staphylococcus intermedius* and *Staphylococcus hyi*cus [6], [10]. *Staphylococcus aureus* forms golden-yellow colonies, often with hemolysis when grown on blood agar plates, however several strains show variants no pigmented. Additionally, *S. aureus* is capable of fermenting manitol aerobic- and anaerobically and can metabolize glucose, xylose, lactose, sucrose, maltose and glycerol. In complex media, *S.*

aureus grows over a wide range of pH (4.8 to 9.4), temperature (25 to 43ºC), and a minimal doubling time of 30 to 40 min [6], [10].

1.2 Genetic characteristics

The genome of *S. aureus* consists of a single circular chromosome (2.7-2.8 Mbp) with a relatively low G+C content [11]. Currently, there are 72 annotated complete whole-genome sequences of *S. aureus* available in public databases (http://www.ncbi.nlm.nih.gov/genome/genomes/154?) reviewed on August 2015). The genome shows two major components: the conserved core genome and the accessory genome.

The core genome is present in all isolates and makes up approximately 75% of a *S. aureus* genome. Core genes are located on the bacterial chromosome, thus usually stable and transferred vertically [12]. The majority of these genes are associated with metabolism and other house-keeping functions. Other genes are related with common functions, but are not essential for growth and survival, including virulence genes not carried by other staphylococcal species, such as toxins, exoenzymes, cell surface binding proteins and the capsule biosynthetic genes [13]. Sequence divergence in the core genome is observed and referred to as “core variable” (CV) genes. These sequence variations may result from single nucleotide polymorphisms (SNPs) to large regions of DNA diversity (ranging in size from a few nucleotides within a gene to several kilobase pairs that include complete or partial genes within operons) [12].

The accessory genome accounts for approximately 25% of *S. aureus* genome, and mainly consists of mobile genetic elements, which are transferred horizontally between strains [13]. These elements include bacteriophages, *S. aureus* pathogenicity islands (SaPI), staphylococcal cassette chromosomes (SCC) regions, plasmids and transposons [12]–[14]. Many of these genetic elements harbour genes with virulence or resistance functions. Hence, the distribution and horizontal spread of these elements might have clinical relevance [13]. Interestingly, virulence genes are likely to be found on phages and SaPI, whereas resistance genes rely on SCCs, plasmids and transposons for transfer [12]. The accessory genes can be transmitted among bacteria by horizontal transfer via three mechanisms: transformation, conjugation and transduction [15]. However, *S. aureus* lack the genes for transformation. Conjugation does not appear to be common, but is an efficient mechanism for gene transfer from other species to *S. aureus*, such as enterococci [16]. Therefore, transduction by bacteriophage is probably the most frequent mechanism of horizontal transfer in *S. aureus*, which therefore plays a crucial role in the evolution of this pathogen [12].

1.3 Colonization

S. aureus is a ubiquitous colonizer of the skin and mucosa of human beings and several animal species. In humans, the anterior nares of the nose are the most frequent carriage site for *S. aureus* [17]. However, some studies reported higher rates of *S. aureus* carriage in the throat compared with the rates in the nose, thus highlighting the importance of including this carriage site when screening the *S. aureus* colonization [18]–[20]. Moreover, this microorganism

can be isolated from other multiple body sites, such as the hands, perineum, forearms, gastrointestinal tract, vagina and axillae, but in a lower frequency [17], [21].

In the 1950s, a clear association between *S. aureus* nasal carriage and infection is supported by the evidence that the nasal *S. aureus* strain and the infecting strain shared the same genotype [22], [23]. It has been shown that nasal carriers have a three to six times increased the risk of acquiring a nosocomial infection with an endogenous *S. aureus* compared to noncarriers and low-level carriers [23], [24]. Decolonization of health-care workers and patients with nasal mupirocin combined with chlorhexidine body washes as a part of a multifactorial approach has been shown to contribute to reduce infection, cross-transmission and be effective to eradicate *S. aureus* from the nose and other body sites [25], [26].

In general, three *S. aureus* nasal carriage patterns can be discriminated: 20% of healthy individuals are persistent carriers, ~30% are intermittent carriers and 50% are non-carriers [21]. Nevertheless, van Belkum *et al*. revealed that apparently, there are only two human types of nasal carriers: persistent carriers and others, because intermittent carriers and noncarriers have similar *S. aureus* elimination kinetics and antistaphylococcal antibody patterns [27]. Furthermore, the load of *S. aureus* is significantly higher in persistent carriers, resulting in an increased risk of infections [28], [29]. Persistent carriers are often colonised by a single strain of *S. aureus* over long periods, up to 10 years, while intermittent carriers carry many different strains over time [30]–[32]. Persistence carriage seems to have a protective effect on the acquisition of other strains and is most

frequently detected in children than in adults, and many individuals change their pattern of carriage between the age of 10 and 20 years [17], [21]. Moreover, *S. aureus* nasal carriage rates vary according to the countries studied. Recently, a cross-sectional survey studying *S. aureus* nasal carriage in nine European countries (Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, the Netherlands and the UK) showed a great variation in the nasal *S. aureus* carriage rates between the participating countries ranging from 12.1% in Hungary to 29.4% in Sweden. In Spain, the prevalence of *S. aureus* nasal carriage was reported to be 17.3% [33].

Nasal colonisation of *S. aureus* needs four main prerequisites: first, *S. aureus* has to come in contact with the anterior nares; second, *S. aureus* adherence to certain receptors in the nasal niche; third, *S. aureus* overcomes the host defences; fourth, *S. aureus* ability to propagate in the nose [17]. The carriage of *S. aureus* could be influenced by several determinants such as host characteristics, bacterial factors and interaction between host and *S. aureus*, causing different carriage rates over the population [17], [21], [24]. Host factors might define carriage or non-carriage status, and probably also whether carriage is persistent or intermittent. It was reported that artificial colonization of non-carriers and persistent carriers showed that non-carriers eliminated the inoculated strains, whereas persistent carriers maintain their original resident *S. aureus* strain [24], [34]. Thus, host characteristics substantially co-determine the *S. aureus* carrier state and this view is supported by the fact that *S. aureus* carriage rates is age-dependent and vary between different ethnic groups, with higher rates in white people and in men [17]. On the other hand, bacterial factors such as the wall teichoic acid (WTA), clumping factor B (*clf*B), the

capsular polysaccharide (*cap*), the iron-regulated surface determinant (IsdA), the surface protein (SasG), autolysins and other adherence and virulence determinants are thought to define which strain is carried, as bacterial competition and biological fitness of bacteria probably influence carriage [34]–[36].

Higher carriage rates have been reported in some groups. In these groups are included: patients with diabetes mellitus (both insulin dependent and non-insulin dependent), patients undergoing haemodialysis or continuous peritoneal dialysis, patients with end stage liver disease, HIV patients, patients with *S. aureus* skin infections and skin disease (i.e, eczema or psoriasis), obesity, history of cerebrovascular accidents and intravenous drugs addicts [34]. Surprisingly, a recent cross-sectional study performed in Spain, showed a low prevalence of MRSA colonization in HIV patients [2]. Recently, livestock farmers, veterinary staff and pet owners have been considered as new risk groups for MRSA colonization [37], [38].

1.4 Pathogenesis

S. aureus has been recognized as a major human pathogen, capable of causing infection when skin and mucous membranes are disrupted [39], [40]. Infection caused by *S. aureus* can range from superficial lesions and skin infections (e.g. folicullitis, cellulitis, mastitis, furuncles, impetigo, wound infections, superficial and deep skin abscesses), to life-threatening diseases (e.g., osteomyelitis, endocarditis, bacteremia, meningitis, pneumonia) and toxinoses (e.g., food poisoning, toxic shock syndrome and scalded skin syndrome) [40]. The success of this microorganism as a pathogen and its

capacity to cause a wide range of infections are the result of being well equipped in surface factors and secreted proteins that mediate host colonization and disease (see Table 1) [1], [41]. Furthermore, global regulatory genes that coordinate the expression of various groups of staphylococcal genes have been identified [1], [40].

Table 1: *Staphylococcus aureus* extracellular factors involved in pathogenesis, and response to global regulatory elements during bacterial growth [1].

					Action of Regulatory Genes[†]			
Gene	*Location*	*Product*	*Activity/Function*	*Timing**	*agr*	*saeRS*	*rot*	*sarA*
Surface proteins								
spa	Chromosome	Protein A	Antiimmune, anti-PMN	exp		‡	+	
cna	Chromosome	Collagen BP	Collagen binding	pxp	0			
fnbA	Chromosome	Fibronectin BPA	Fibronectin binding	exp				+
fnbB	Chromosome	Fibronectin BPB	Fibronectin binding	exp				+
clfA	Chromosome	Clumping factor A	Fibrinogen binding	exp	0			
clfB	Chromosome	Clumping factor B Lactoferrin BP	Fibrinogen binding Lactoferrin binding	exp	0		+	0
Capsular polysaccharides								
cap5	Chromosome	Polysaccharide capsule type 5	Antiphagocytosis?	pxp	+			+
cap8	Chromosome	Polysaccharide capsule type 8	Antiphagocytosis?	pxp	+			
Cytotoxins								
hla	Chromosome	α-hemolysin	Hemolysin, cytotoxin	pxp	+	+	−	‡
hlb	Chromosome	β-hemolysin	Hemolysin, cytotoxin	pxp	+	+	−	‡
hld	Chromosome	δ-hemolysin	Hemolysin, cytotoxin	xp	+	0		+
hlg	Chromosome	γ-hemolysin	Hemolysin, cytotoxin	pxp	+		−	‡
lukS/F	PVL phage	PVL*	Leucolysin	pxp	+		−	
Superantigens								
sea	Bacteriophage	Enterotoxin A	Food poisoning, TSS	xp	0			
seb	SaPI3[§]	Enterotoxin B	Food poisoning, TSS	pxp	+			‡
sec	SaPI4[§]	Enterotoxin C	Food poisoning, TSS	pxp	+			
sed	Plasmid	Enterotoxin D	Food poisoning, TSS	pxp	+			
eta	ETA phage	Exfoliatin A	Scalded skin syndrome	pxp	+			
etb	Plasmid	Exfoliatin B	Scalded skin syndrome	pxp	+			
tst	SaPI1,2,bov1[§]	Toxic shock toxin-1	Toxic shock syndrome	pxp	+			‡
Enzymes								
SplA-F	Chromosome	Serine protease-like	Putative protease		+		−	
ssp	Chromosome	V8 protease	Spreading factor	pxp	+	0		−
aur		Metalloprotease (aureolysin)	Processing enzyme?	pxp	+			−
sspB		Cysteine protease	Processing enzyme?		+		−	
scp		Staphopain (protease II)	Spreading, nutrition	pxp	+			−
geh	Chromosome	Glycerol ester hydrolase	Spreading, nutrition	pxp	+	0	−	‡
lip		Lipase (butyryl esterase)	Spreading, nutrition	pxp	+	0		‡
fme	Chromosome	FAME	Fatty acid esterification	pxp	+			‡
plc		PI-phospholipase C		pxp	+			
nuc	Chromosome	Nuclease	Nutrition	pxp	+	+		
hys	Chromosome	Hyaluronidase	Spreading factor	xp	‡			
coa	Chromosome	Coagulase	Clotting, clot digestion	exp		+	+	+
sak	Bacteriophage	Staphylokinase	Plasminogen activator	pxp	+	0		

*Timing: *xp*, throughout exponential phase; *exp*, early exponential phase only; *pxp*, postexponential phase; *0*, no effect of gene on. Expression: +, upregulated; −, downregulated.
[†]*agr*, accessory gene regulator; *saeRS*, *S. aureus* exoproteins; *rot*, repressor of toxins; *sarA*, *Staphylococcus* accessory regulator; FAME, fatty acid modifying enzyme; PMN, polymorphonuclear neutrophil.
[‡]Controversial.
[§]*SaPI*, *S. aureus* pathogenic island.
BP, binding protein.

1.4.1. Regulation

At least three major operons are involved to adjust gene expression to specific environmental conditions: *agr* (accessory gene regulator), the *sar* (staphylococcal accessory regulator) and the *sae* (staphylococcal accessory element) loci [38], [42], [43]. Additional regulatory systems influencing virulence genes expression have been described: *arlR* and *arlS* (autolysis-related locus sensor), *rot* (repressor of toxins), *mgr* (multiple gene regulator) and *sigB* (staphylococcal alternative sigma factor B) [44]–[47].

The staphylococcal *agr* locus encodes a quorum sensing (QS) gene cluster of five genes (*hld*, *agrB*, *agrD*, *agrC*, and *agrA*) that works as a classical two-component signalling module. This system reacts to bacterial density, allowing the preferential expression of surface adhesins during the exponential phase of growth (low cell density) and switching to the expression of exoproteins during the postexponential and stationary growth phases (high cell density) [48], [49]. This coordinated shift in expression patterns brings a strategic switch to decrease unnecessary metabolic demands [50]. Specific variations in all three AgrB, AgrD, and AgrC proteins (i.e., the processors-transporter, the auto-inducing peptide precursor, and the receptor, respectively) have resulted in four *agr* groups in *S. aureus* [51]. Loss-of-function mutations in *agr* locus confer alterations in the expression of autolysins and hemolysins and can affect bacterial phenotypes, including pathogenicity [2], [29], [30], [40], [52]. *S. aureus agr* dysfunction (more-frequent defects in production of delta hemolysin) has been associated with persistent bacteremia and with attenuated vancomycin bactericidal activity [33], [37], [45], [52].

Of note, not all *S. aureus* strains produce the same virulence factors, which in some cases could be clonally related [53], [54]. This clonal relation could be established when comparing *agr* groups with other epidemiological markers. Most of the *S. aureus* that produces the TSST-1 toxin or the Panton-Valentine toxin belongs to *agr* group III; vancomycin-intermediate strains tend to be associated to *agr* group II; and the exfoliative toxin A (ETA)-producing strains belong to *agr* group IV. Hence, global regulators were originally meant to control the expression of useful metabolic genes [1].

Essentially, any *S. aureus* genotype can become a life-threatening human pathogen, but strains from some clonal lineages are more virulent than others [55].

1.4.2. Virulence determinants

S. aureus has an extensive variety of virulence factors, with both structural and secreted products (see Figure 2) playing an important role in pathogenesis of infection. These virulence factors can be grouped into functional categories: (i) attachment and adherence to host tissues; (ii) evasion and destruction of host defences; (iv) invasion and penetration tissue; (v) persistence in the host [54].

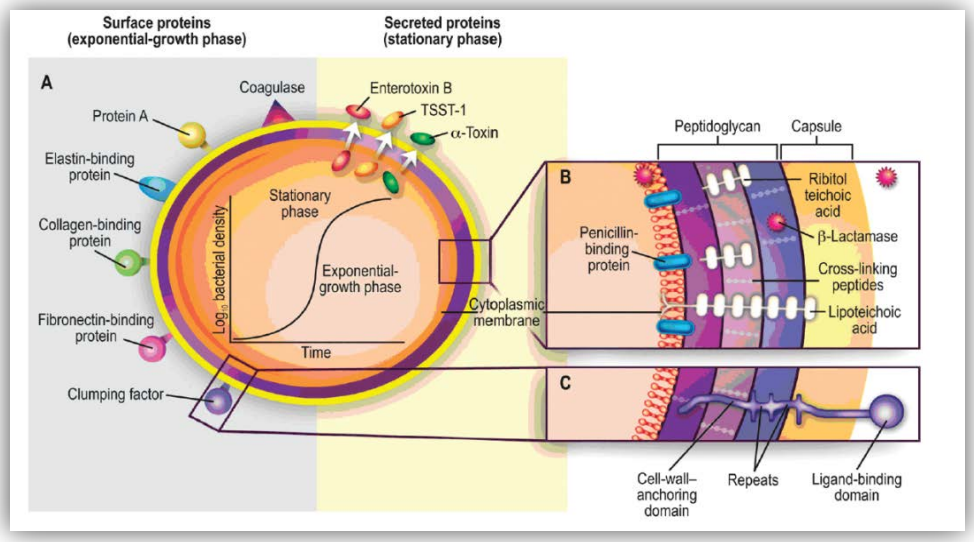


Figure 2: Pathogenic factors of *S. aureus*, with structural and secreted products both playing roles as virulence factors. Panel A shows the surface and secreted proteins. Panels B and C show cross sections of the cell envelope. TSST-1 denotes toxic shock syndrome toxin 1 (from [40]).

- **Bacterial attachment**

Surface proteins: *S. aureus* adhesion to host tissues including host extracellular matrix proteins such as fibrinogen, collagen, fibronectin and elastin is mediated by bacterial cell-wall-associated protein called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) [56]. This initial step is crucial in colonization and invasive diseases such as endovascular infections, bone and joint infections, and prosthetic-device infections[54], [57]. MSCRAMMs belong to the largest class of surface proteins and are covalently anchored to the cell wall peptidoglycan [58]. The main MSCRAMMs found in *S. aureus* are fibrinogen-binding proteins C, D and E (SdrC, SdrD snd SdrE), collagen-binding protein (Cna), fibronectin-binding proteins A and B (FnBPA and FnBPB), clumping factors A and B (ClfA and ClfB), elastin binding protein S (EbpS) [59]. Besides adhesion to and invasion of

host cells and tissues, these surface proteins have been implicated in evasion of immune responses and biofilm formation [58].

- **Bacterial persistence**

Biofilm accumulation: Once *S. aureus* attaches to host proteins or prosthetic materials, it is able to grow and persist in various ways. Biofilm (slime) formation is one of the possible ways to persist by evading host defences and antimicrobials. Bacterial biofilm is a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced. Biofilm formation is associated with production of a substance named polysaccharide intercellular adhesion (PIA), which is synthesized by an operon called *ica* composed of a regulator (*icaR*) and biosynthetic (*icaADBC*) genes [60].

Small colony variants: Another strategy used by *S. aureus* for persisting in the host and resist antibiotic therapy is the formation of small colony variants (SCVs) [61]. SCVs constitute a slow-growing auxotrophic subpopulation of bacteria with distinctive phenotypic and pathogenic traits. They are usually recovered from patients with antibiotic-refractory infections such as cystic fibrosis patients (CF), patients with skin and foreign-body related infections and osteomyelitis [62]. SCVs are often tolerant or resistant to a number of other antibiotics in addition to the selecting drug, including β-lactams and glycopeptides [63]. Based on the knowledge that SCVs invade and persist in eukaryotic cells, prolonged treatment including antimicrobial agents with intracellular antistaphylococcal activity appears appropriate [1], [61].

- **Evasion of the host mechanism defences**

Capsule: One of the *S. aureus* main defences that help it to evade the host immune system during an infection is the production of an antiphagocytic microcapsule, among which 11 serotypes have been reported. Most *S. aureus* clinical isolates produce either a serotype 5 or 8 capsular polysaccharide [64], [65].

Protein A: In addition, *S. aureus* (but not the coagulase-negative staphylococci) have a surface protein called protein A (belongs to the MSCRAMMs family) that is covalently linked to peptidoglycan layer and has antiphagocytic properties due to its ability to bind the Fc portion of immunoglobulin [40], [66].

Immune modulators: *S. aureus* is also able to produce small solubles molecules that specifically interact with crucial elements on the immune system such as chemotaxis inhibitory protein of staphylococci (CHIPS) and staphylococcal complement inhibitor (SCIN) [67], [68]. CHIPS and SCIN are both efficient modulators of neutrophil chemotaxis, phagocytosis and killing, their early expression is necessary for efficient modulation of the early immune response. [69]–[75]. SCIN and CHIPS are located on an 8-kb region at the conserved 3' end of β-hemolysin (hlb) – converting bacteriophages (βC-ϕs) that also encodes the genes for staphylokinase (SAK). SAK has been described as a modulator of different parts of the immune system: directly destroys defensins and has antiopsonic activities [76], [77]. These innate immune modulators form an immune evasion cluster (IEC) that is easily transferred among *S. aureus* strains by a diverse group of βC-ϕs [78]. Recently, IEC is gaining interest due its

specificity for human-adapted *S. aureus* strains and could be a good genetic marker to predict human or animal origin of a given isolate [79], [80]. Moreover, another *S. aureus* virulence determinant named extracellular adherence protein (Eap) could act in concert with CHIPS to inhibit neutrophil recruitment to the site of infection [66].

Phenol-soluble modulins (PSMs): PSMs have recently emerged as novel toxin family defining the virulence potential of highly aggressive *S. aureus* isolates. PSMs were named according to the grouping of PSMs into the smaller PSMs α-type and the larger β-type group. These secreted staphylococcal peptides have a remarkable ability to recruit, activate and lyse human neutrophils, thus eliminating the main cellular defence against *S. aureus* infection. The strong impact that PSMs, and especially α-type PSM (PSMα), have on the development of acute forms of *S. aureus* disease identifies these peptides as promising targets for drug development [81]. PSMα are produced at higher concentrations by standard community-associated MRSA (CA-MRSA) strains and probably contributes to the enhanced virulence of those strains compared to hospital-associated MRSA (HA-MRSA) strains [82].

Citotoxins: Finally, *S. aureus* can produce a high number of citotoxins such as α-, β-, γ- and δ-hemolysins, leukocidins (LukD, LukE and LukM) and the Panton-Valentine leukocidin (PVL) that cause erythrocytes and leukocytes destruction by the formation of beta-channel pores in the cytoplasmatic cell membrane [7], [66], [83]. In contrast to α-, β-, γ- and δ-hemolysins that are present in most *S. aureus* isolates, PVL is secreted only by strains lysogenized with bacteriophage carrying the structural genes for PVL [1], [84], [85]. α-

hemolysin (or α-toxin), but not PVL, was found to play an essential role for the pathogenesis of staphylococcal pneumonia [86].

PVL was first described in 1984 by Van de Velde but was first associated with skin and soft tissue infections (SSTIs) in 1932 by Panton and Valentine. PVL belongs to the described family of synergohymenotropic toxins comprising two-component pore-forming proteins [87]. The two nonassociated active proteins are designated S (from slow-eluted) and F (from fast-eluted) which are encoded by two contranscribed genes, *lukF-PV* and *lukS-PV* and carried by several temperate bacteriophages [85], [88]. Similar to other leukocidins, PVL has the ability to form pores on polymorphonuclear leukocytes (PMNs) membranes, monocytes and macrophages, leading to their lysis or apoptosis, in a PVL concentration-depending manner [89], [90]. Several epidemiological studies have reported the strong link between PVL and CA-MRSA disease, considering PVL one of the molecular markers for CA-MRSA. Although PVL is a useful marker for strains with the potential to cause severe *S. aureus* infections (e.g. chronic/recurrent SSTI and necrotizing pneumonia in children and young adults), discordant results from mouse infection models suggest that this toxin is not the major determinant of disease caused by CA-MRSA strains [89], [91]–[94]. The emergence and spread of virulent clones expressing PVL is an additional cause of concern. Until now, PVL-positive MRSA are found in several different clone (STs) and genetic backgrounds (CC) such as CC1, CC5, CC8, CC22, CC30, CC59, CC80, CC88, ST72, ST93, ST154, ST398 and ST772 [95].

- **Invasion and penetration of tissue**

Enzymes: During infection, *S. aureus* produces various enzymes, such as proteases, lipases, nucleases, staphylokinase and elastases that facilitate the evasion and destruction of host tissues and metastasize to other sites [6], [40]. However, the most important are:

- Catalase: protects intraphagocytic microbes by destroying hydrogen peroxide produced by the phagocyte and contribute to the survival of *S. aureus* in the presence of *Streptococcus pneumoniae* [96], [97].
- Hyaluronidase - breaks down hyaluronic acid, a major component of the extacellular matrix of human tissues and helps in spreading it [98].
- Penicilinase – is a secreted enzyme that hydrolyses penicillin and other penicillinase–suceptible compounds into inactive penicilloic acid [1].

- **Toxin-mediated disease and sepsis**

Cell wall and α-toxin: *S. aureus* is capable of producing septic shock by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid and α-toxin are virulence factors that may play a role in causing this clinical syndrome. Peptidoglycan and lipoteichoic acid could have endotoxin-like activity, stimulating the release of citotoxines by macrophages, activation of complement, and aggregation of platelets. These structures can be hidden from host recognition, by producing antiphagocytic components such as a capsule or protein A [1], [40], [99].

Superantigens (SAgs): Additionally, some *S. aureus* strains can also produce SAgs, resulting in various toxin-mediated diseases, such as food

poisoning and toxic shock syndrome (TSS) [83], [100]. SAgs toxins are a class of powerful immune-stimulatory molecules that possess the ability to interact simultaneously with the major histocompatibility complex-(MHC) class II molecules and T-cell receptors, inducing T-cell proliferation and massive burst in cytokine release [1], [101]. These toxins include toxic shock syndrome toxin-1 (TSST-1), and staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI) [83], [100], [102]. TSS is an acute and potentially fatal illness that is characterized by fever, diffuse erythematous rash, hypotension, desquamation that can lead to multiple organ failure and lethal shock. This syndrome has been reported in two clinical forms: menstrual TSS and nonmenstrual TSS. TSST-1 is responsible for nearly all cases of menstrual-associated TSS and nonmenstrual-associated staphylococcal TSS is normally associated with TSST-1, staphylococcal enterotoxin SEB and SEC [83], [100]. Moreover, SEs are the causative agents of staphylococcal food poisoning, which is a major concern in public health worldwide [103]. Several SAgs genes are encoded by staphylococcal pathogenicity islands (SaPI), prophages and plasmids [104]. Recently, a few reports demonstrated that the presence of different SAgs is associated with clonal complex rather than with invasive disease and these toxins are critical in the causation of lethal sepsis, infective endocarditis, and kidney infections [105]–[107].

Exfoliatins: Some strains also produce epidermolysins or exfoliative toxins (ETA, ETB, ETC and ETD, proteases that act by cleaving peptide bonds between the extracellular domains of desmoglein 1, a transmembrane protein that forms part of desmosomes that bind the epithelial cells [108]–[110]. These

toxins are capable of causing scalded skin syndrome or bullous impetigo, which clusters in newborns and infants less than 1 year old and rarely in adults [1].

- **Virulence factors with poorly defined role in virulence**

Arginine catabolic mobile element (ACME): Another element that has been associated to play a role in the pathogenesis of MRSA, particularly in CA-MRSA, is the type I ACME, which is a 31-kb mobile genetic element (MGE). ACME was first described in the epidemic USA300 clone (ST8 and carry a type IV staphylococcal cassette chromosome (SCC*mec*). This element contains two genes clusters: *arc*, encoding the arginine deiminase pathway, and *opp3*, encoding an oligopeptide permease system [111]–[113]. Arginine deiminase activity, which produces ammonia and ATP, it seems to facilitate colonization by neutralizing the acid environment (pH 4.2-5.9) on the skin and producing energy. In addition, inhibits nitric oxide production a key molecule used by the human innate and adaptive host defences. Oligopeptide permeases can be associated to a wide array of functions, including uptake of short peptides as nutrients, quorum sensing, chemotaxis, cell adhesion, and resistance to antimicrobial peptides [90], [111], [114]. The biochemical functions of the ACME *opp* and *arc* operons and a possible role of ACME in colonization, remains to be determined [90]. ACME is adjacent to SCC*mec* and mobilized by the same recombinase (*ccrAB*) that mobilizes SCC*mec*. Physical linkage between ACME and SCC*mec* suggests that selection for pathogenicity and selection for antibiotic resistance are interconnected. In community setting with low antibiotic selection pressure, ACME could enhance growth, survival, and dissemination of USA300 clone [112]. In addition to ST8-IV, ACME has been found in different

clonal backgrounds such as ST5-II, ST59-IV, ST97-V, ST1-IV, ST5-IV, ST239-III, also two ST8 MSSA isolates and in some species of coagulase-negative staphylococci, where it seems to be more prevalent [111], [115]–[121].

Coagulase: Coagulation is a primitive innate defence mechanism against microbial pathogens that traps and immobilizes invading bacteria in a clot and is the target of bacterial immune evasive strategies [122]. The bacterial pathogen *S. aureus* secretes coagulases polypeptides that bind to and activate prothrombin, thereby converting fibrinogen to fibrin and promoting the clotting of plasma or blood. Two proteins promote coagulation during the host infection, coagulase (Coa) and von Willebrand factor binding protein (vWbp). Staphylococcal binding to fibrinogen or fibrin is an important characteristic of disease pathogenesis, enabling the formation of abscesses, bacterial persistence in host tissues and enables the pathogen to cause lethal sepsis [122], [123]. However, the molecular basis of protective immunity by Coa and vWbp specific immune responses remains to be evaluated [123]. As referred before, coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory [40].

Bacteriocins: Bacteriocins (Bac) are ribosomally synthesised antimicrobial peptides that are not lethal to the producer cells but toxic or antibiotic to other bacteria [124], [125]. The synthesis of a number of Bac has been shown to be growth-phase dependent and appears to be under the control of a large family of two-component signal transduction systems [125]–[127]. Several strains of *S. aureus* carry the entire biosynthetic operon for the production of a functional lantibiotic-type Bac, designated Bsa (bacteriocin of *S. aureus*). Bsa could help highly virulent CA-MRSA strains such as ST8 and ST80 genetic backgrounds,

compete with other colonizing flora and increase the chance of infection with this strains [11], [90], [128].

2. Antibiotic resistance in *Staphylococcus aureus*

Nowadays, one of the major concerns of clinicians is the emergence of pathogens resistant to multiple antibacterial agents causing *S. aureus* infections in the clinical setting.

Before the antibiotic era, the mortality rate of *S. aureus* bacteremia was extremely high, exceeding 80% [129]. In the early 1940s, with the introduction into clinical practice of the first beta-lactam antibiotic named penicillin, the mortality and morbidity decreased considerably [130]. However, soon after its introduction, bacteria resistant to penicillin began to evolve and spread [131]. Worryingly, the deployment of any novel antibiotic has been followed by the evolution of clinically significant resistance to that antibiotic in as little as a few years (see Figure 3).

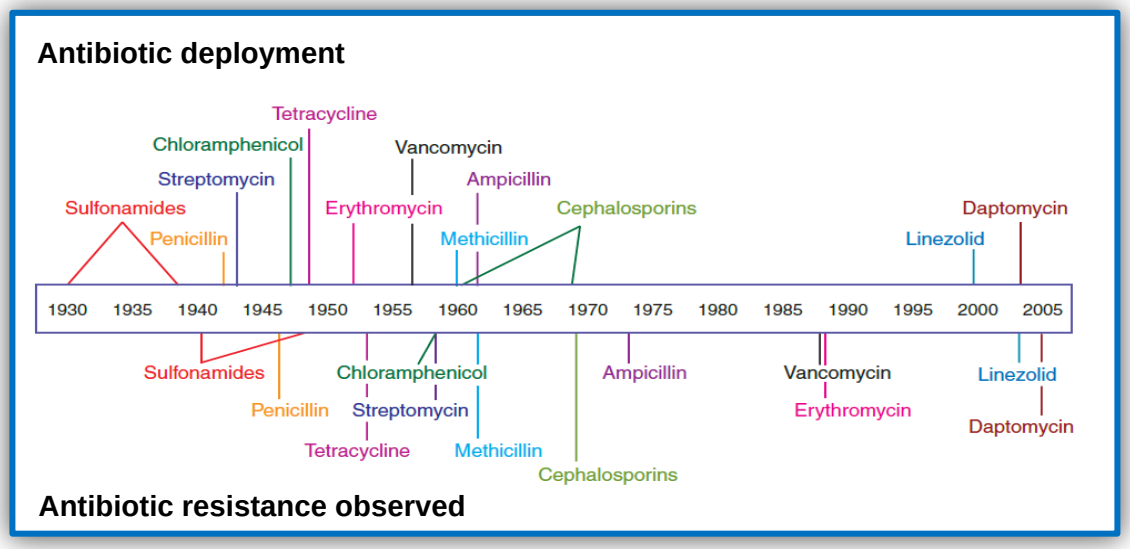


Figure 3: Timeline of antibiotic introduction in the clinical practice and the emergence of antibiotic resistance (taken from [132]).

This represents an increasing global problem that seriously complicates the treatment of bacterial infections. Antibiotic use in human and animal medicine, to a lesser extent, in agriculture clearly selects the bacterial populations

(pathogenic and/or commensal endogenous microbiota of human and animals) [133].

The clinical relevant antibiotics may be classified in five categories according to the cellular mechanisms they target: i) cell wall synthesis (e-g. beta-lactams and glycopeptides), ii) protein synthesis (e.g. aminoglycosides, tetracyclines, macrolides, lincosamides, chloramphenicol, mupirocin and fusidic acid), iii) DNA replication (e.g. quinolones), iv) RNA synthesis (e.g. rifampin), v) folic acid metabolism (e.g. sulphonamides and trimethoprim) (see Figure 4) [134].

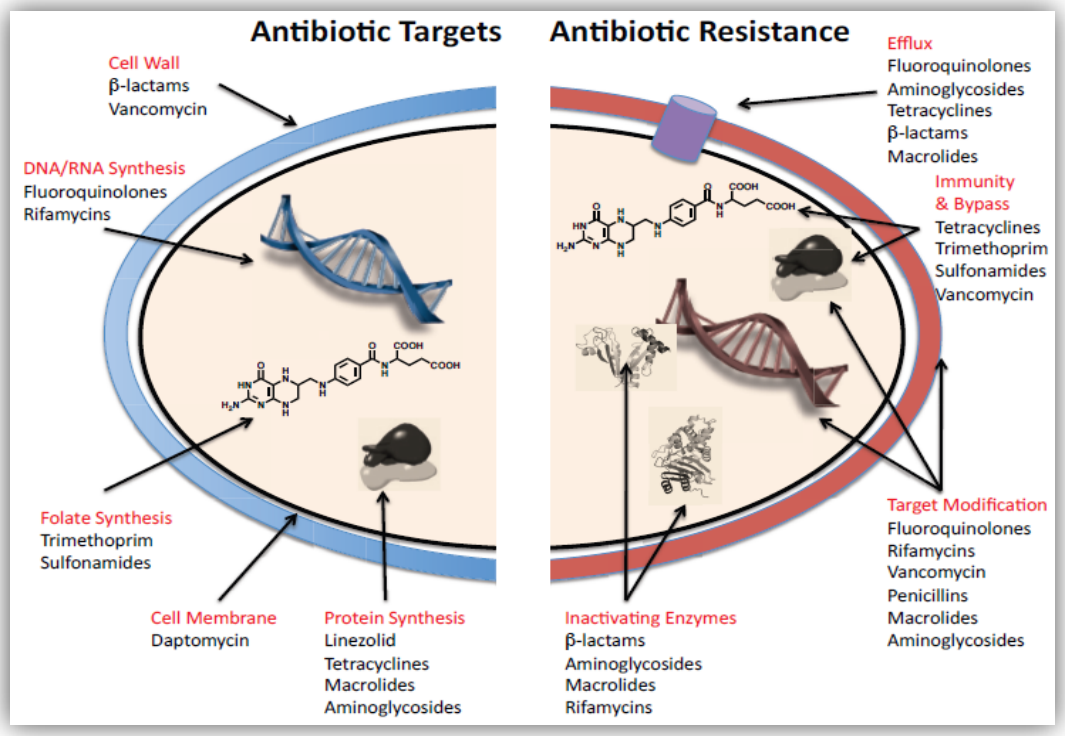


Figure 4: Antibiotic targets and mechanisms of resistance (taken from [134]).

The basic mechanisms by which microorganisms developed antibiotic resistance can be classified into three different classes: i) prevention of access to target (reduced permeability, active efflux, or reduced influx); ii) drug inactivation (destruction or inactivation of the antibiotic, so it fails to bind the

target), and iii) target alteration (overproduction of target and modification/alteration, avoiding the antibiotic binding) (see Figure 4 and Table 2). Overall, bacteria can possess one or several of these mechanisms simultaneously [134], [135].

Table 2 summarizes the most important resistance mechanisms and resistant genes associated with MRSA.

Table 2: Antimicrobial agent classes, targets, mechanisms of action, mechanisms of resistance and resistance genes (adapted from [136], [137]).

Class (examples)	**Target**	**Mechanism of action**	**Resistance type**	**Mechanism of resistance**	**Resistance genes**	**Genetic basis**
Beta-lactams (Penicillins, Caphalosporins, Carbapenems, Monobactams)	Cell wall synthesis	Bind to penicillin-binding proteins (PBPs) and inhibit the transpeptidation step in the peptidoglycan synthesis, stimulate autolysins	Inactivation (hydrolysis) New target with reduced affinity	Beta-lactamase Additional and altered PBPs	*blaZ* *mecA*	P, T (Tn552) C (SCC*mec*)
Glycopeptides (Vancomycin, Teicoplanin)	Cell wall synthesis	Inhibit transglycosylation and transpeptidation steps in peptidoglycan synthesis – bind to D-Ala-D-Ala	Target alteration Target overproduction	Altered peptidoglycan cross-link target (substitute D-Ala-D-lactate for D-Ala-D-Ala) Excess of peptidoglycan	*vanA* Unknown	T (Tn*1546*) Probably C
Aminoglycosides (Gentamicin, Trobamycin, Neomycin, Kanamycin, Streptomycin)	Protein synthesis (30S)	Inhibit 30S ribosomal subunits	Inactivation (acetylation, phosphorylation, adenylation)	Aminoglycoside-modifying enzymes AAC6'/APH2'' ANT4',4'' APH3' ANT3''	 *aacA-aphD* *aadD* *aphA-3* *aadE*	 T (Tn*4001*) P (pUB110) T (Tn*5404*, Tn*5405* located into P or C) T (Tn*5404*, Tn*5405* located into P or C)
Tetracyclines (Tetracycline, Tigecycline)	Protein synthesis (30S)	Inhibit protein synthesis (30S) Disrupt bacterial membrane	Ribossomal protection Active efflux	Production of proteins that bind to the ribossome and after the conformation of the active site New membrane transporters	*tetM* *tetK*, *tetL*	C P (T181), P
Macrolides/Lincosamides/Streptogramins (B) (Erythromycin, Clindamycin, Quinupristin, Dalfopristin)	Protein synthesis (50S ribosomal subunit)	Inhibit 50S ribosomal subunits	Target alteration	23S rRNA methylase	*erm(A), erm(B), erm(C)*	T (Tn*554)* P (pI258 type) P (pE194 type)
Macrolides/Streptogramins (B) (Erythromycin, Quinupristin, Dalfopristin)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Active efflux	ABC transporter	*msrA*	P (pUL5050)
Streptogramins (B) Quinupristin, Dalfopristin)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Inactivation (hydrolysis)	Hydrolase	*vgb*	P
Streptogramins (A) Quinupristin, Dalfopristin)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Active efflux Inactivation (acetylation)	ABC transporter Acetyltransferases	*vga, vgaB* *vat, vatB*	P P

Table 2: Antimicrobial agent targets, mechanisms of action, mechanisms of resistance and resistance genes (*cont.*)

Class (examples)	**Target**	**Mechanism of action**	**Resistance type**	**Mechanism of resistance**	**Resistance genes**	**Genetic basis**
Lincosamides (Clindamycin)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Inactivation (hydrolysis)	*o*-nucleotidyltransferase	*linA*	P (pIP855 type)
Phenicols (Chloramphenicol)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Inactivation (acetylation) Methylation Active efflux	Acetyltransferase CAT rRNA methylase Membrane transporter system	*cat* *cfr* *fexA*	P (pUB112) P T (*Tn558*)
Fusidanes (Fusidic acid)	Protein synthesis (elongation factor G)	Inhibit protein synthesis (elongation factor G) Protection of the drug target site	Target alteration Acquisition of *fusB* family proteins	Mutation leading to reduced binding to active site (s) Acquisition of *fusB*, *fusC*	*fusA*, *rplF* *fusB*, *fusC*	C P, T-like or SaPI
Mupirocin (Mupirocin)	Protein synthesis (isoleucyl-tRNA-synthetase)	Inhibit isoleucyl-tRNA-synthetase)	Target modification	Resistant isoleucyl-tRNA-synthetase	*mupA*	P
Quinolones (Ciprofloxacin, Norfloxacin, Levofloxacin)	DNA replication	Bind DNA gyrase	Target alteration Active efflux	Mutations in gyrase Mutations in topoisomerase Multidrug efflux system	*gyrA/B* *grlA/B* *norA*	C C C
Rifamycin (Rifampicin)	RNA synthesis	Bind to beta-subunit of bacterial RNA polymerase	Target alteration	Mutations leading to reduced binding to RNA polymerase	*rif*	C
Lipopetides (Daptomycin)	Cell membrane charge and cell wall synthesis	Inhibit lipotheichoic acid biosynthesis; disrupt bacterial membrane	Target alteration	Mutations in multiple chromosomal loci changing the cell membrane charge - decreasing drug binding	*mprF*, *yycG*, *rpoB*, *rpoC*	C
Oxazolidinones (Linezolid)	Protein synthesis (50S)	Inhibit 50S ribosomal subunits	Target alteration Methylation	Mutations in the V domain of the 23S rRNA Modification of ribosomal proteins L3 and L4 RNA methylation	G2447T, T2500A, G2576T *rplC* , *rplD* *rlmN* *cfr*	C C P P
Fosfomycin (Daptomycin)	Peptidoglycan synthesis	Inhibit peptidoglycan synthesis (inactivation of MurA)	Target alteration	Transporter mutation	*fosB*	P
Sulfonamides/Trimethoprim (Trimethoprim, Trimethoprim-Sulfamethoxazole)	Folic acid metabolism Tetrahydrofolate production	Metabolic Pathways folic acid metabolism Inhibit enzymes responsible for tetrahydrofolate production	Target alteration	Mutation or recombination of genes encoding DHPS and DHFR Acquisition of new low affinity DHFR genes Promoter mutation leading to overproduction of DHFR	*dhps*, *dhfr* *dhfrS1*, *dhfrS2*, *dhfrS3* *dfrA*, *dfrD*, *dfrG*, *dfrK*	P P P

AAC, aminoglycoside acetyltransferase; APH, aminoglycoside phosphotransferase; ANT, aminoglycoside nucleotidyltransferase; ABC, ATP-binding cassette; CAT, chloramphenicol acetyltransferase; P, plasmid; T, transposon; C, chromosome.

2.1. Resistance to beta-lactam antibiotics: methicillin

Beta-lactam antibiotics are important in the treatment of *S. aureus* infections. Beta-lactam antibiotics act by acylation (inactivating) the active site of the transpeptidase of the penicillin-binding proteins (PBPs), inhibiting cell wall synthesis [138].

The beta-lactam resistance of MRSA strains results from the expression, in addition to the four native PBPs, of a fifth low-affinity PBP named PBP2a [139]. Nevertheless, other types of resistance to beta-lactams have been reported in *S. aureus*, namely the presence of chromosomal mutations, the overexpression of the beta-lactamase or the overexpression of PBPs [140]–[145].

2.1.1. Emergence and evolution of methicillin-resistance

Resistance to beta-lactams was first described for penicillin in 1940 and is mainly caused by the *blaZ* gene encoding production of beta-lactamases, which hydrolytically destroy beta-lactams [146]. The *blaZ* gene can be located chromosomally or on plasmids [147]. This type of penicillin resistance in *S. aureus* may thus emerge via two mechanisms: spread of resistant clones or through horizontal dissemination of mobile elements containing the *bla*Z gene. Location of the resistance determinants on transferable elements generally promotes efficient spread [148].

After the spread of *S. aureus* strains resistant to penicillin through the acquisition of a beta-lactamase, methicillin was introduced in Europe in 1959, which was not degraded by beta-lactamases. However, once again, it was not long before the first staphylococci isolates exhibiting resistance to methicillin

were reported and methicillin-resistant *S. aureus* (MRSA) emerged [146], [149], [150].

Since then, nosocomial MRSA infections emerged, though infrequently, worldwide. In the late 1980s and the early 1990s, MRSA gradually increased in frequency and became a serious pathogen in hospital throughout the world, the so-called health care or hospital-associated MRSA (HA-MRSA) [151], [152]. Today, MRSA has risen to one of the most frequent causes of hospital infections worldwide [152]. In some hospitals, over half of the *S. aureus* infections are MRSA. The prevalence varies greatly depending on the country and even from one hospital to another.

2.1.2. *mecA* and *mecC* genes

The intrinsic resistance to all beta-lactam antibiotics delivered by PBP2A, results from the fact that this protein in contrast to the four native PBPs of *S. aureus* has very low affinity for beta-lactam antibiotics. Therefore, its transpeptidase domain remains active in the presence of otherwise inhibitory concentrations of beta-lactams assisted by the transglycosylase domain of native PBP2, ensuring the cell wall biosynthesis [139], [153], [154].

PBP2a is encoded by the *mecA* gene, a 2.1Kb DNA fragment of unknown origin, which is carried by a heterologous mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) [155], [156]. The origin of *mecA* remains unclear. A *mecA* homologous gen was detected in *Staphylococcus sciuri* sharing a high amino acid sequence similarity (88%) with the *mecA* gene of *S. aureus*. Moreover, the comparison of transpeptidase

domain and transglycosylase domain of the *S. sciuri mecA* revealed a similarity of 96% and 80%, respectively, with the MRSA *mecA* gene [157], [158].

Recently, a divergent *mecA* homologue, showing 70% DNA sequence identity with *mecA* named *mecC* has been described in MRSA from humans and diverse animal species (LGA251). This *mecA* homologue, originally named $mecA_{LGA251}$ and later designated *mecC,* shows lower levels of resistance to methicillin and cefoxitin than *mecA*, is negative by PCR assay for *mecA* and fails the slide agglutination test for PBP2a [159]–[161]. Several studies have reported the presence of this gene in distinct *S. aureus* genetic lineages from both animal and human origin with a wide geographical distribution in Europe [162]–[164].

2.1.3. Staphylococcal cassette chromosome *mec* (SCC*mec*) element

SCC*mec* integrate into the *S. aureus* chromosome at the 3' end of *orfX*, an open reading frame (ORF) of unknown origin coding for a methyltransferase of RlmH type, located near the origin of replication [165]–[167]. SCC*mec* is composed by: (i) the *mec* gene complex including *mecA* and its regulators; (ii) the *ccr* gene complex containing site –specific recombinases – cassette chromosome recombinases (*ccr*) and (iii) the presence of three flanking regions, the joining (J) regions [168]. The *mec* complex is composed of the *mecA* gene, intact or truncated *mecA* regulators, *mecI* (repressor) and *mecR1* (sensor inducer). Recently, *mecR2* was also identified as a regulator of *mec* through binding of the methicillin repressor *mecI* (see Figure 5) [169], [170].

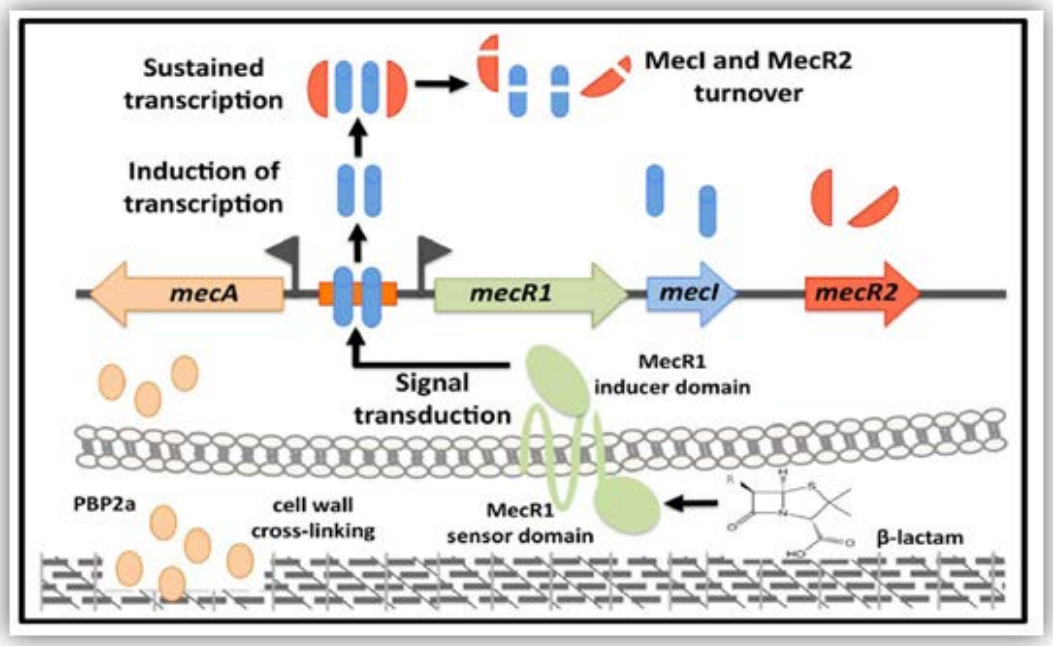


Figure 5: Model for the *mecA* induction by MecR1-MecI-MecR2. In the presence of a beta-lactam antibiotic, MecR1 is activated and rapidly induces the expression of *mecA* and *mecR1-mecI-mecR2*. The anti-repressor activity of MecR2 is essential to sustain the *mecA* induction since it promotes the inactivation of MecI by proteolytic cleavage. In the absence of beta-lactams, MecR1 is not activated and a steady state is established with stable MecI-dimers bound to the *mecA* promoter and residual copies of MecR1 at the cell membrane (taken from [170]).

So far, five major classes of *mec* complexes (A, B, C1, C2, and E) have been described in *S. aureus* according to its structure (see Table 3) [167], [168], [171]–[174]. The *ccr* complex is composed by the *ccr* genes that encodes recombinases of the invertase resolvase family and are responsible for the excision/integration of SCC*mec* from the chromosome [156], [174]. Currently, five *ccr* gene complexes have been described: types 1 to 4, carrying two genes, *ccrA* and *ccrB* with four allotypes (*ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*); and type 5 carrying a single allotype, *ccrC* (see Table 3*)*. Besides the *mec* and *ccr* gene complexes, the SCC*mec* is composed by three J regions (J1, J2 and J3)

(previously named “junkyard” regions), which constitute non-essential components of the cassette that may carry antibiotic and heavy metal resistance determinants [168]. J1 is the region between the chromosomal left junction and the *ccr* complex; J2 the region between the *ccr* complex and the *mec* complex and J3 the region between the *mec* complex and the chromosomal right junction. Overall, SCC*mec* structural organization may be summarized as containing a J1-*ccr*-J2-*mec*-J3, where the variations in the J regions (within the same *mec*-*ccr* combination) are used for defining SCCmec subtypes [168], [175]. Currently, eleven different types of SCC*mec* elements (types I-XI) have been described in *S. aureus*, ranging in size from 20.9 to 66.9Kb (see Table 3). Moreover, a total of 19 subtypes have been reported, namely from SCC*mec* type I (IA), type II (IIA, IIvar, IIb, IIA-E), type III (IIA and IIIB) and from SCC*mec* type IV (IVa-d, IVg-j) [168], [172], [176]–[182].

SCC*mec* types IV, V, VI and VII were defined by the presence of beta-lactams resistance determinants. In contrast, types I, II, III and VIII carry additional resistance determinants for other antibiotics and heavy metals. This last resistant profile could be conferred by the presence of integrating plasmids, such as pI258 coding for resistance to mercury, pT181 coding for tetracycline resistance and pUB110 encoding for resistance to kanamycin, tobramycin and bleomycin; and transposons like *Tn554* carrying the *ermA* gene that confers inducible resistance to macrolides, lincosamides and streptogramins (MLS_B phenotype) [174]. The acquisition by *S. aureus* of these SCC*mec* types gives rise a multidrug resistant (MDR) phenotype in a single event.

Table 3: SCC*mec* types identified in *S. aureus* (I to XI) (adapted from http://www.sccmec.org/Pages/SCC_TypesEN.html).

SCCmec type	SCC*mec* size (Kb)	*ccr* gene complex	*mec* genes complex	Prototype strains
I (1B)	34.3	type 1 (A1B1)	B	NCTC10442, COL
II (2A)	53.0	type 2 (A2B2)	A	N315, Mu50, Mu3, MRSA252, JH1, JH9
III (3A)	66.9	type 3 (A3B3)	A	85/2082
IV (2B)	20.9-24.3	type 2 (A2B2)	B	CA05/MW2, 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, EMRSA-15, JCSC6668, JCSC6670
V (5C1)	28.0	Type 5 (C1)	C2	WIS (WBG8318), TSGH17, PM1
VI (4B)	20.9	Type 4 (A4B4)	B	HDE288
VII (5C1)	33.3	Type 5 (C1)	C1	JCSC6082
VIII (4A)	33.7	Type 4 (A4B4)	A	BK20781
IX (1C2)	44.3	Type 1 (A1B1)	C2	JCSC6943
X (7C1)	51.5	Type 7 (A1B6)	C1	JCSC6945
XI (8E)	29.4	Type 8 (A1B3)	E	LGA251

2.2. Resistance to non beta-lactam antibiotics

During the past years, the emergence of MRSA strains resistant to several antimicrobial classes in addition to beta-lactams antibiotics has complicated the treatment of *S. aureus* infections [183]. This fact has resulted in the increasing use of glycopeptides such as vancomycin and teicoplanin and other antimicrobial agents such as tetracyclines, clindamycin, quinolones and fusidic acid for the treatment of MRSA infections. However, MRSA have progressively become resistant to a number of antimicrobials, including clindamycin and tetracycline [183], [184]. In response to these challenges, new antimicrobials have been developed including the streptogramins (quinupristin/dalfopristin), the oxazolidinones (linezolid), telavancin ceftaroline and, more recently, the cyclic lipopeptides (daptomycin) [183].

The European antimicrobial resistance surveillance network (EARS-net) reported in 2013 that 81.4% of the invasive MRSA isolates registered in 28 European countries showed resistance to fluoroquinolone. Moreover, low resistance levels to rifampicin (6.7%) and linezolid (1.2%) were observed [185].

2.2.1. Vancomycin

Vancomycin is a glycopeptide antibiotic used as a first-line treatment against severe MRSA infections [186]. However, the first MRSA isolate with decreased susceptibility to vancomycin (minimum inhibitory concentration [MIC] of 4-8mg/L) also known as vancomycim intermediate-resistant *S. aureus* (VISA), was reported in Japan in 1997 [187]. Additional cases were subsequently reported from other countries including Spain [188]–[192].

Two types of vancomycin resistance were identified in clinical isolates of *S. aureus* [193]. One type was reported in the VISA strains, which have MICs to vancomycin of 8-16mg/L that also includes a pre-VISA stage of resistance - heterogeneously resistant strains (hetero-VISA) [187]. Hetero-VISA strains are phenotypically susceptible to vancomycin by routine laboratory methods but contain vacomycin-intermediate subpopulations that are usually present frequencies of 10^{-6} to 10^{-4} [194]. The reduced susceptibility to vancomycin seems to result from changes in peptidoglycan biosynthesis. VISA strains synthetise additional quantities of peptidoglycan that result in irregularly shaped thickened cell wall. There is also decreased cross-linking of peptidoglycan strands which leads to the exposure of more D-Ala-D-Ala residues. Therefore, there are more D-Ala-D-Ala residues available to bind and trap vancomycin. This bound acts as a further obstacle to drug molecules reaching their target on

the cytoplasmatic membrane (see Figure 6A) [195]. The second type of vancomycin resistance probably results from the conjugal transfer of the *vanA* operon from a vancomycin *Enterococcus faecalis*. This operon is located on a plasmid and encodes a sex pheromone that is produced by *S. aureus*, suggesting a potential facilitator of conjugal transfer. These isolates have MICs ≥128 mg/L and are caused by the replacement of the terminal peptide D-Ala-D-Ala by D-Ala-D-Lac. Furthermore, the new dipeptide has dramatically reduced affinity for vancomycin. In the presence of vancomycin, the novel cell wall precursor is synthesized, allowing continued peptidoglycan assembly (see Figure 6B) [195], [196].

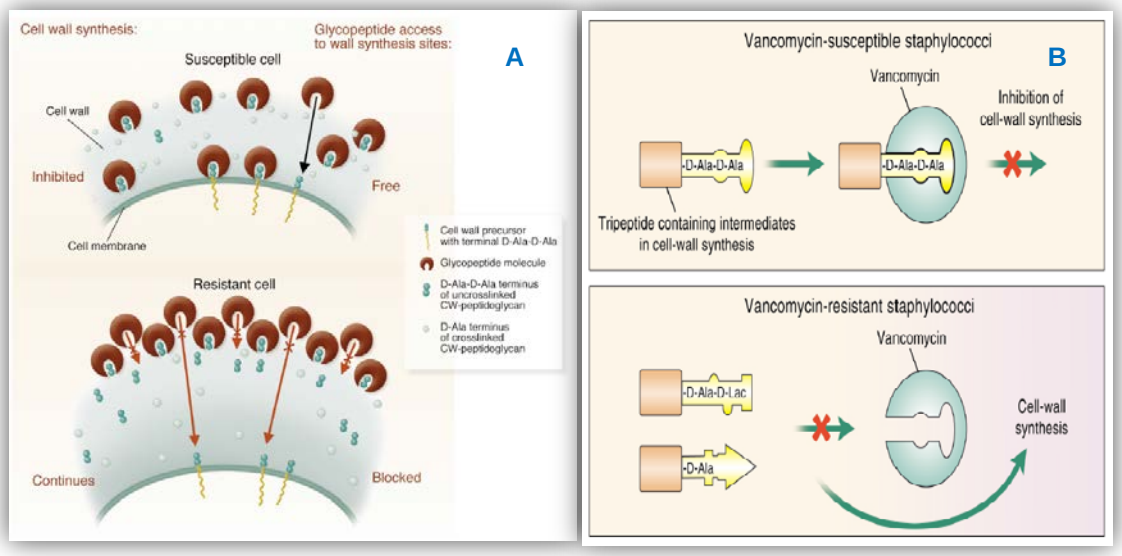


Figure 6: Mechanisms of *S. aureus* resistance to vancomycin: A) VISA strains; B) VRSA strains **(**taken from [195]).

2.2.2. Daptomycin

Daptomycin is a cyclic lipopeptide antibiotic derived from the fermentation of *Streptomyces roseosporus*, with a rapid bactericidal activity against Gram-positive pathogens, including MRSA and approved for the treatment of skin

infections, bacteremia and right-sided endocarditis [197]. Despite the cell membrane has usually been pointing as a daptomycin's central target, recent studies have shown that it also causes alterations in the activity of enzymes involved in cell wall synthesis, therefore causing a dramatic cell wall and membrane defects [198]. Consequently, cell membrane depolarization and permeabilization is accompanied by leakage of small ions such as potassium, resulting in bacterial cell death. This process requires the presence of calcium, which may increase its amphipathicity and lower its negative molecular charge at neutral pH. In interaction with calcium, daptomycin resembles cationic antimicrobial peptide (CAMPs) [199], [200]. Its activity is concentration dependent, and higher doses are therefore expected to provide increased activity and reduce infection inoculums more quickly [201].

Both the US Food and Drug Administration (FDA) and the Clinical Laboratory Standards Institute (CLSI) have established the daptomycin susceptibility breakpoint for *S. aureus* at an MIC ≤1 mg/L and consider strains that have an MIC above 1mg/L as being non-susceptible [202].

Evidence-based guidelines for the management of patients with MRSA infections recommended daptomycin as a first-line treatment for SSTIs, infective endocarditis and bacteremia. In contrast, it is not useful for low respiratory tract infections due to its inactivation by pulmonary surfactant [203].

The emergence of daptomycin non-susceptible *S. aureus* during therapy has been described in several reports [204]–[207]. Most of these patients had deep-seated infections associated with a high burden of infecting organisms,

commonly with the presence of a biomedical device or dead tissue with poor blood perfusion [201].

The mechanisms of resistance to daptomycin are quite diverse and complex, involving perturbations predominantly in the cell membrane, but also in the cell wall thickening, changes in membrane lipid composition, drug binding and alterations in the surface charge [199].

Daptomycin resistant strains often exhibit progressive accumulation of single nucleotide polymorphisms in a relatively limited group of genes in *S. aureus* involved in the regulation of bacterial membrane surface charge, such as *mprF* [encoding a membrane lysylphosphatidylglycerol (LPG) synthetase] and *yycFG* (encoding a histidine kinase) [208]. Mutations in subunits of RNA polymerase (*rpoB* and *rpoC*) have also been identified in later stages of the progression of daptomycin resistance in laboratory-derived strains [209].

The mutations most frequently identified have been in the *mprF* gene. This gene is responsible for the lysinylation of phosphatidylglycerol (PG) to generate a more positively charged LPG (mediated by a C-terminal lysinylation domain) and translocates LPG to the outer layer of the cell membrane (mediated by the N-terminal translocation domain) [210]. “Gain of function” *mprF* mutations can be associated with increased surface positive charge, conferring resistance to daptomycin due to electrostatic repulsion and decreased membrane binding. Nevertheless, the relationship between *mprF* gene function and associated cell wall changes in daptomycin resistant isolates has not been completely clarified to date [199], [211].

Daptomycin and cationic antimicrobial peptides (CAMPs) resistance could be also mediated by *Dlt*ABCD operon, which is responsible for D-alanylating wall teichoic acids (WTA) and contributes to the positive surface charge. Inactivation of *S. aureus dlt* genes caused a reduction in the D-alanine content of WTA, as well as to increased susceptibility to CAMPs [212], [213].

Additionally, mutations in cardiolipin synthetase (*csl2*) and phosphatidyglycerol synthase (*psgA*) genes, which are responsible for the production of the anionic membrane phospholipids, have also been related with daptomycin resistant strains (see Figure 7) [214].

It appears that prior vancomycin exposures may well provide the development of subsequent daptomycin-resistant MRSA strains. Those strains lack mutations in any of the membrane phospholipid biosynthesis genes. The mechanism of resistance might be due to impaired diffusion through thicker, less cross-linked cell walls, possible reduction in muramic acid O-acetylation, and possible changes in membrane charge [199].

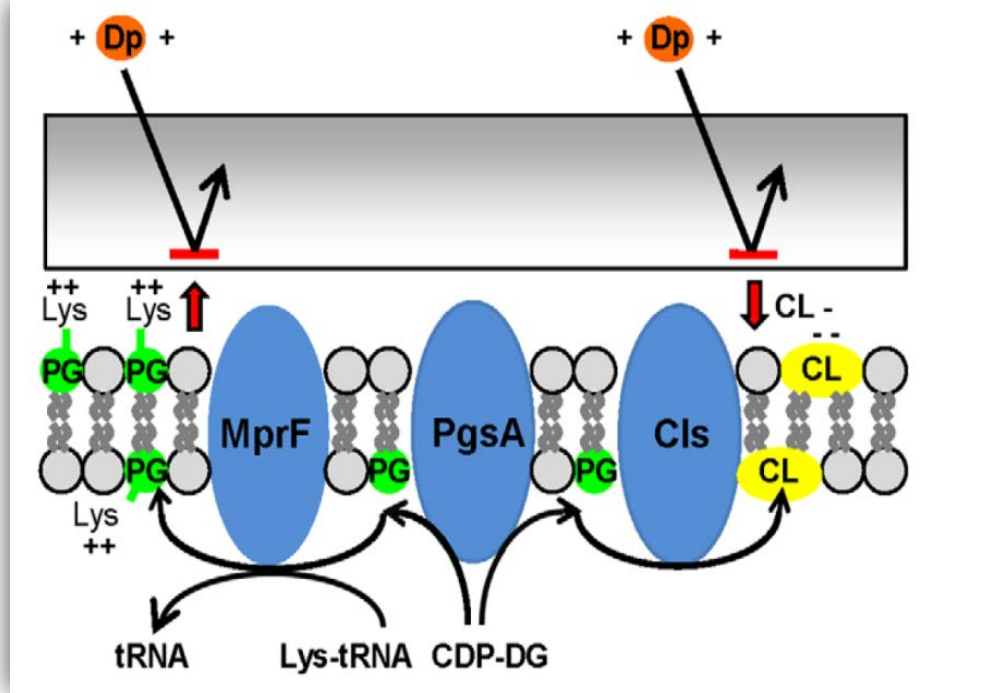


Figure 7: A hypothetical schematic diagram for the functional effect of the mutations in *mprF*, *pgsA* and *cls2* genes. *mprF* mutations lead to an increase in lysinylation of phosphotidylglycerol (PG) to form L-PG, and an increase in translocation of this positively charged L-PG to the outer leaflet of the membrane, leading to electrorepulsion of daptomycin. In isolation, or in concert with *mprF* mutations, mutations in *cls2* may lead to altered membrane charge or effect binding of daptomycin to the membrane. Finally, PgsA is important in the initial step of phospholipid biosynthesis, converting CDP-diacylglycerol (CDP-DG) into PG (taken from [214]).

2.2.3. Linezolid

Linezolid is the first oxazolidinone antibiotic that has been introduced into the medical practice to treat infections caused by various Gram-positive microorganisms, including MRSA [215], [216]. This bacteriostatic agent binds to the 50S subunit of the bacterial ribosome via interaction with the 23S rRNA, thereby blocking protein synthesis (see Figure 8) [215], [217], [218].

Clinical Laboratory Standards Institute (CLSI) have established the linezolid susceptibility breakpoint for *S. aureus* at an MIC ≤4 mg/L and consider strains that have an MIC above 8 mg/L as being non-susceptible [202]. Resistance to linezolid in *S. aureus* is not frequent in Spain, though it has increased during the last decade [219], [220].

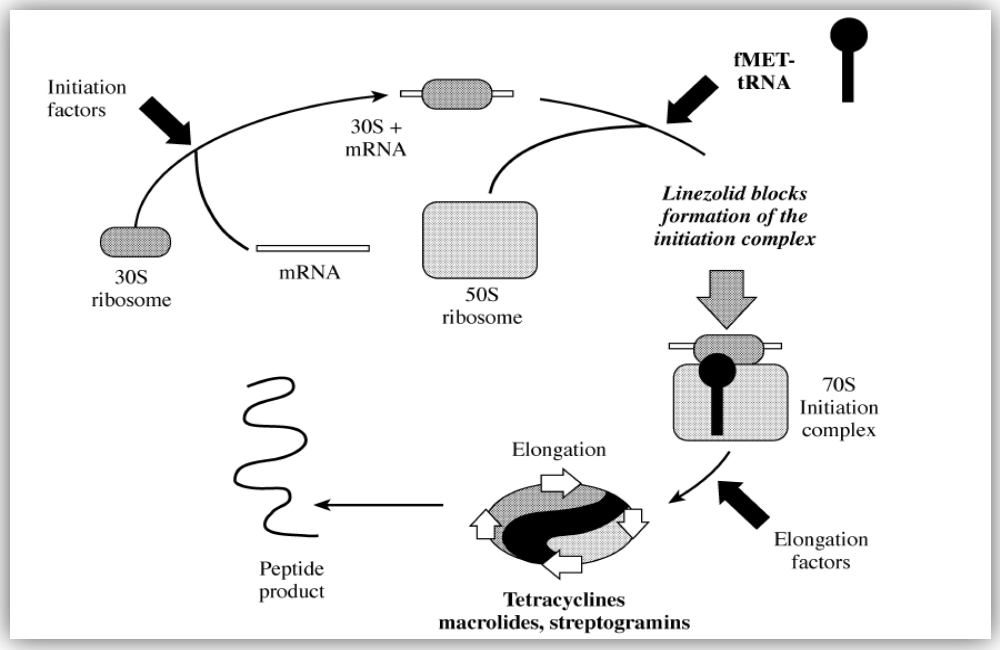


Figure 8: Mode of action of the oxazolidinones. Oxazolidinones combine with the 50S ribosomal unit, preventing it from complexing with the 30S subunit, mRNA, initiation factors and formylmethionyl-tRNA. Consequently, no functional initiation complex is formed, and protein synthesis is halted. Most other protein synthesis inhibitors block peptide elongation (taken from [216]).

Since the introduction of linezolid in the clinical practice, several mechanisms of linezolid-resistance have been described. The most frequent mechanism of resistance is mediated by mutations in the V domain of the 23S rRNA, particularly G2447T, T2500A and G2576T [215], [216]. Due to the presence of multiple copies of the 23S rRNA gene, a relationship between the number of mutated genes and the level of resistance is well established, and known as “gene dosage” [221]. MIC increments are progressive, and MICs of

such mutants are usually 4 to 8 mg/L compared with a baseline of 2 mg/L. Another mechanism involved in linezolid resistance is the modification of ribosomal proteins L3 and L4 encoded by *rplC* and *rplD* genes, respectively. Some of the mutations found in these genes are concurrent with mutations in the V domain of the 23S rRNA [222]. Finally, RNA methylation by two different methyltransferases has been related to linezolid resistance: RlmN – a codon insertion in the methyltransferase gene *rlmN* reduces linezolid susceptibility in clinical *S. aureus*, and a methyltransferase encoded by the *cfr* (chloramphenicol-florfenicol resistance) gene [223], [224]. The *cfr* gene was originally described in a bovine *Staphylococcus sciuri* isolate [225]. It is often located in a plasmid which may be horizontally transferred between staphylococci and confers cross-resistance to linezolid and four other classes of antimicrobial agents [phenicols, lincosamides, pleuromutilines, and streptogramin A (PhLOPS phenotype)] [226], [227]. Some outbreaks of *cfr*-mediated linezolid-resistant strains have been reported in the literature such as the outbreak described in Spain by Morales *et al*. [219]. In this case, the epidemic strain was involved in surgical site infections; ventilator-assisted pneumonia; and primary bacteremia in an intensive care unit, affecting 12 patients.

Evidence-based guidelines for the management of patients with MRSA infections recommended linezolid as a first-line treatment for SSTIs, persistent bacteremia, vancomycin treatment failures, bone and joint infections, infections of the central nervous system and pneumonia. In contrast, it should not be used if there is concern for infective endocarditis or endovascular source of infection

but may be considered in children whose bacteremia rapidly clears and is not related to an endovascular focus [203].

2.3. New therapeutic strategies

In recent years, development of new antimicrobials not adequately addresses the problems posed by antibiotic resistance among multidrug-resistant bacteria [228].

Several attempts to identify the correct antigen or antigen combination and relying on the production of a protective antibody against *S. aureus* have been made, however it remains unclear how to successfully formulate a successful vaccine and to whom it should be deployed [229], [230]. Alternative approaches have been proposed to target virulence of pathogens instead of their growth to generate new antimicrobial agents, thereby reducing or slowing the selection for resistance [231]–[233]. Anti-virulence compounds acting against *S. aureus* biofilm formation and targeting two essential *S. aureus* virulence factors like alpha-hemolysin (Hla) and phenol-soluble modulins (PSMs), have been developed [234]. Furthermore, natural products with antimicrobial activity against MRSA have been also explored. For instance, honey has demonstrated antimicrobial activity against MRSA forming biofilm and synergistic effects in combination with rifampicin for the treatment of chronic wound infections caused by MRSA [235]. Other natural compounds such as Marinopyrrole A (identified from a species of marine-derived streptomycetes) that has significant affinity for plastic have potential as a potent anti-MRSA agent in cutaneous, intracatheter or antibiotic-lock applications [236].

3. Epidemiology of methicillin-resistant *Staphylococcus aureus*

As referred before, after the introduction of methicillin into clinical practice to treat infections caused by penicillin-resistant *S. aureus*, the first MRSA strain was reported from United Kingdom (UK) in 1961 [150]. During the following 1980s, MRSA have acquired multidrug resistance traits and has spread to other European countries. During this time, MRSA infections had been largely confined to hospitals and other healthcare facilities (so called hospital-associated MRSA, HA-MRSA) [152]. However, in the late 1990s, virulent community-acquired MRSA (CA-MRSA) emerged in the community and later on in the healthcare facilities suggesting that certain clones have the ability to cross barriers between hospitals and the community [40], [195], [237]–[239]. While the distribution between hospital and community MRSA became blurred, MRSA started to emerge from another origin – livestock-associated MRSA (LA-MRSA). In spite of *S. aureus* being associated with animals for several years, MRSA transmission from animal origin into humans poses a serious public health problem [240]–[249].

3.1. Methicillin-resistant *S. aureus* clonal lineages: evolution and global dissemination

Two theories were proposed to explained the origins of the major contemporary MRSA clones: (i) the “single-clone theory” suggests that all MRSA were descendant from a common MRSA ancestor, resultant from a single SCC*mec* introduction event into a methicillin-susceptible *S. aureus*

(MSSA) strain; (ii) the “multiclone theory”, which is nowadays the most widely accepted, proposes that SCC*mec* has been introduced several times into various *S. aureus* lineages [151], [250]–[252]. Crisóstomo and colleagues approached questions related to the origin and spread of MRSA by studying the genetic background of MSSA and early MRSA strains isolated in the 1950/60s in Denmark and UK, where the first MRSA were identified and stored. This study noticed a gradual accumulation resistance determinants in MSSA paralleling the antimicrobial introduction in therapeutics and suggested that these MSSA strains (belonging to CC8) had a similar genetic background to the early and contemporary MRSA (ST250-I, the Archaic clone). Thus, the Archaic clone represented the progeny of an *S. aureus* strain that must have been served as one of the first *S. aureus* recipients of the methicillin resistance determinant in Europe [253]. Another study including *S. aureus* blood isolates that were collected in Denmark in the 1960s shows the same findings [250].

Because of the emergence and spread of multidrug-resistant MRSA worldwide, over 3000 MRSA isolates from different regions of the world has been studied by the international surveillance system CEM/NET. This surveillance study evidenced the spread of six major clones, namely the Iberian (ST247-IIA), Brazilian (ST239-IIIA), Hungarian (ST239-III), New York/Japan (ST5-II), Pediatric (ST5-VI) and EMRSA (ST36-II) clones [254]. Minor clones such as EMRSA-15 (ST22-IV) and Berlin (ST45-IV) mainly detected in the UK and Germany, showed epidemic potential [255]–[258].

Enright *et al*. studied a wide collection of MRSA and MSSA isolated in 20 countries between 1961 and 1999, by MLST and SCC*mec* typing and defined

that the major MRSA clones could be grouped into five CCs: CC5, 8, 22, 30 and 45. Additionally, according to their data, ST8-MSSA lineage was the ancestor of the Archaic clone (ST250-MRSA-I), because these STs differ only in a single mutation at *yqiL* locus [151]. Several evolutionary models were constructed for each of the five major CC, based on point mutations in housekeeping genes, recombination processes and acquisition of SCC*mec* element [151], [250], [259].

Besides ST250-MRSA-I, there are other successful MRSA lineages within CC8 such as the ST247-MRSA-I (Iberian clone), the ST239-MRSA-III (Brazilian and Hungarian clone), ST254-MRSA-IV (Hannover clone), ST8-MRSA-II (Irish-1 clone) and one clone that is one of the major source of community-acquired infections in the USA, Canada and Europe, the ST8-MRSA-IV PVL positive USA300 [113], [151], [259]. ST239-III is one of the most epidemic MRSA clones and also the first bacterial hybrid identified in nature that arose by the homologous recombination of a 557Kb fragment of the ST30 chromosome with the chromosome of a ST8 strain. Thus, this clone represents an important and distinct branch of CC8 [260].

In addition to CC8, CC5 originated from an ancestral ST5-MSSA, represented the most diversified lineages of MRSA and contained the most pandemic clones. The ancestor ST5-MSSA diverged into major actual clonal lineages, as the ST5-MRSA-II (New York/Japan clone), the ST5-MRSA-IV/VI (Pediatric clone) and ST228-MRSA-I (Southern German clone) [259]. Moreover, it was reported that variations of the successful CC5 emerged by temporal and geographic independent SCC*mec* acquisitions [251].

CC22, CC30 and CC45 lineages represent less diversified lineages of MRSA strains and they have been mostly reported in Europe [261]–[265]. In CC22 lineage, ST22-MSSA was the ancestor for ST22-MRSA-IV named EMRSA-15 clone, depending on the geographical origins of the isolates In CC45, ST45-MRSA-IV (Berlin clone) emerged from the ancestor ST45-MSSA [259]. In CC30, the ST30-MSSA is the putative ancestor of two currently divergent clonal types: the community acquired ST30-MRSA-IV PVL positive (Southwest Pacific clone) and the ST36-MRSA-II (the hospital acquired, EMRSA-16) [259], [266].

Based on evolutionary models, SCC*mec* seems to be a crucial element on the MRSA evolution; hence, MRSA has emerged at least 20 times upon SCC*mec* acquisition. The acquisition of SCC*mec* by MSSA clones was four times more common than the replacement of one SCC*mec* with another. Remarkably, SCC*mec* type IV was found in twice as many clones as any other SCC*mec* with another [251], [259].

3.2. Hospital-associated MRSA

Hospital-associated (HA-) MRSA are commonly associated with bacteremia, surgical wound, pneumonia, urinary tract, prosthetic-device and catheter-related infections [267]. Defined risk factors for HA-MRSA infection include prolonged hospitalization or residence at a nursing home, patients undergoing dialysis, use of indwelling catheters, prior antimicrobial therapy, Intensive Care Unit exposure, surgical procedures, previous MRSA infection or colonization and close proximity to an impatient colonized with MRSA [268]. The growing impact of medical, devices, older age, comorbidities of patients

and antibiotic overuse seems to contribute to the burden of MRSA infections [269].

Despite the lack of information from some countries, there is a gradient of prevalence, giving low values (5-10%) in Canada and Saudi Arabia and higher values (10-25%) in Tunisia, Venezuela, Mexico, Morocco and India. The prevalence increases significantly in the United States, Chile, Brazil, South Africa and Australia, among others, reaching up to 50%. Some countries such as Colombia and Japan are among the countries that exceed the value of 50% [152], [270].

In Europe, according to the last report (2013) from the European Antimicrobial Resistance Surveillance Network (EARS-NET), the MRSA prevalence in invasive disease is 18% and seems to be decreasing or at least stabilizing, however with proportions ranging from 0% (Iceland) to 64.5% (Romania). Two countries (Iceland and Norway) reported resistance percentages below 1%, five countries (Sweden, Netherlands, Denmark, Finland and Estonia) reported 1-5%, five countries (Latvia, Luxembourg, Slovenia, Austria and Lithuania) reported 5-10%, 10 countries (Germany, Czech Republic, United Kingdom, Poland, Belgium, France, Bulgaria, Ireland, Hungary and Croatia) reported 10-25%, five countries (Slovakia, Cyprus, Italy, Greece and Portugal) 25-50% and two countries (Malta and Romania) reported above 50%. Several factors could influenced this MRSA percentages across the countries such as: (i) the different antimicrobial use policies, (ii) the subsequent antimicrobial selective pressure and (iii) different infection control and

prevention program, like Search and Destroy policies in some north European countries [185].

In Spain, the prevalence of MRSA ranging from 1.5% in 1986 to 22.6% in 2013, although a significantly decreasing trend was reported for the period 2010 to 2013 (see Figure 9) [185], [271].

A few data are available concerning Africa and Asia MRSA resistance. The prevalence of MRSA is lower than 50% (range, 16-55%) in most of the African countries during 2002-2011 [272]. In Asia, the MRSA proportion in the hospitals ranging from 28% (in Hong Kong and Indonesia) to >70% (in Korea) in the early 2010s [273].These high levels of MRSA resistance could be due to the lack of restrictive measures on the use of antibiotics [274]; http://www.who.int).

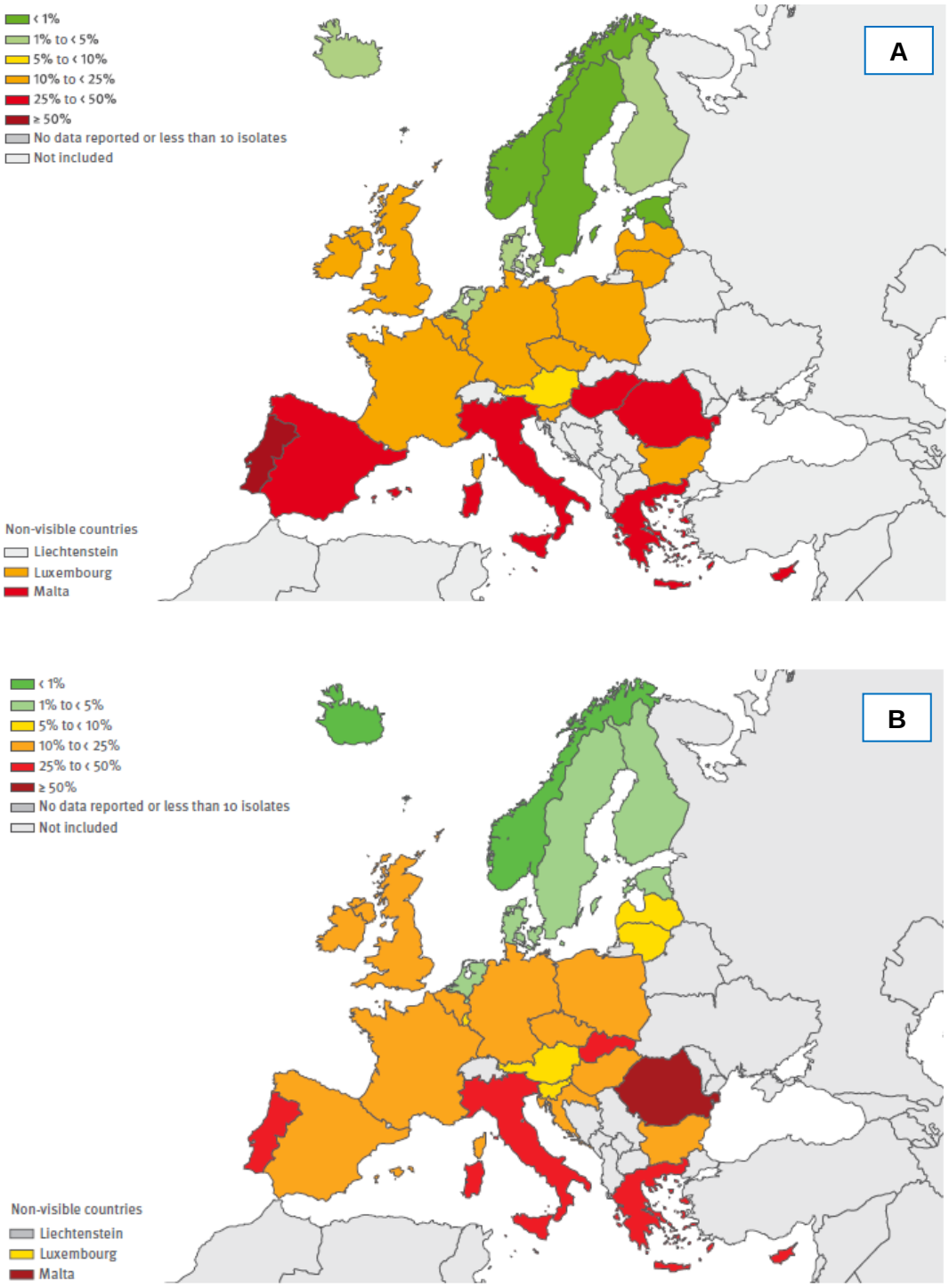


Figure 9: Percentage (%) of invasive MRSA isolates, by country, European Union/European Economic Area countries: A) 2010; B) 2013. Reproduced from [185].

In table 4 are represented the major HA-MRSA clones currently disseminated worldwide.

Table 4: Molecular characteristics and geographic distribution of the predominant HA-MRSA clones (adapted from [275]–[277]).

HA-MRSA clone	CC	ST	SCC*mec*	*spa* type	Geographic spread
Archaic	8	250	I	t008, t009, t194	Ast, Can, Den, Ger, Swi, Uga, UK, USA
Iberian	8	247	I	t008, t051, t052, t054, t200	Aus, Bel, Cro, Cze, Den, Fin, Fra, Ger, Hun, Ita, Isr, Net, Nor, Pol, Por, Slo, Spa, Swe, Swi, UK, USA
Brazilian/Hungarian	8	239	III	t030, t037, t234, t387, t388	Alg, Arg, Ast, Aus, Bra, Can, Chi, Chn, Cze, Den, Fin, Ger, Gre, Hun, Ind, Ids, Kor, Mon, Net, Nor, Par, Pol, Por, Sin, Slo, Spa, Sri, Swe, RoG, Rus, Saud, Tai, UK, Uru, USA, Vie
Irish-1	8	8	II	t008, t024, t064, t190, t206, t211	Ast, Can, Ire, UK, USA
UK EMRSA-2/-6 (USA500)	8	8	IV	t008, t024, t064, t190, t206, t211	Ast, Aus, Bel, Can, Chn, Den, Fin, Fran, Ger, Hun, Ire, Isr, Net, Nor, Swi, Tai, UK, USA
New York/Japan (USA100)	5	5	II	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Ast, Bel, Can, Chn, Den, Fin, Fra, Ger, Hun, Ire, Isr, Jap, Kor, Mex, Nor, Por, SA, Saud, Sin, Swe, Swi, Tai, Uru, UK, USA
Paediatric (USA800)	5	5	IV/V	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Alg, Arg, Ast, Aus, Bra, Col, Den, Fin, Fra, Ger, Gre, Jap, Kor, Nor, Par, Pol, Por, Slo, Spa, Tai, Swe, Uru, UK, USA
Southern-Germany	5	228	I	t001, t023, t041, t188, t201	Aus, Bel, Den, Fin, Ger, Hun, Ita, Slo, Spa, Swi
EMRSA-15	22	22	IVh	t005, t022, t032, t223, t309, t310, t417, t420	Ast, Aus, Bel, Can, Chn, Cze, Den, Fin, Ger, Hun, Ire Kuw, Mal, Nor, NZ, Sin, Spa, Swe, UK
EMRSA-16 (USA200)	30	36	IV	t018, t253, t418, t419	Ast, Aus, Bel, Can, Den, Fin, Ger, Gre, Ire, Mex, Nor, Spa, Swe, Swi, UK, USA
Berlin (USA600)	45	45	IV	t004, t015, t026, t031, t038, t050, t065, t204, t230, t390	Arm, Ast, Aus, Bel, Chm, Den, Fin, Ger, Hun, Isr, Net, Nor, Spa, Swe, Swi, USA

Alg, Algeria; Arg, Argentina; Arm, Armenia; Ast, Australia; Aus, Austria; Bel, Belgium; Bra, Brazil; Can, Canada; Chi, Chile; CHn, China; Col, Colombia; Cro, Croatia; Cze, Czech Republic; Den, Denmark; Fin, Finland; Fra, France; Ger, Germany; Gre, Greece; Hun, Hungary; Ind, India; Ids, Indonesia; Ire, Ireland; Isr, Israel; Ita, Italy; Jap, Japan; Kor, Korea; Kuw, Kuwait; Mex, Mexico; Mon, Mongolia; Net, Netherlands; Nor, Norway; NZ, New Zealand; Par, Paraguay; Pol, Poland; Por, Portugal; RoG, Republic of Georgia; SA, South Africa; Saud, Saudi Arabia; Sin, Singapore; Slo, Slovenia; Sri, Sri Lanka; Spa, Spain; Swe, Sweden; Swi, Switzerland; Tai, Taiwan; Uga, Uganda; UK, United Kingdom; Uru, Uruguay; USA, United States of America; Vie, Vietnam.

Finally, several reports of infections caused by HA-MRSA clones that occurred outside hospitals have been published. One of the most successful HA-MRSA clones that are frequently disseminated in the community is the EMRSA-15 clone, which adapts easily to different hosts including animals [37], [87], [278]. This clone was found in colonization and infection in the community in different locations, namely UK, Belgium, Italy and Portugal [237], [279]–[283]. Furthermore, in Japan, the New York/Japan clone has also been spreading the community among paediatric outpatients and healthy children, in episodes of necrotizing pneumonia and even on public transport [284]–[286]. In East Asia, HA-MRSA ST239-III and ST5-II clones are also spreading in the community [287]. Interestingly, these HA-MRSA clones which harboured larger SCC*mec* elements (SCC*mec* type II and SCC*mec* type III) with additional antimicrobial resistance traits, did not restricted them from spreading into the community, where much lower antibiotic pressure exist.

3.3. Community-associated MRSA

The perception that MRSA infections are confined to the nosocomial setting changed in the mid- and late 1990s with the increasing number of MRSA infections in the community in otherwise healthy people [87], [288]–[290].

The first case of community-associated MRSA (CA-MRSA) infection was reported among Aboriginal patients in remote communities from Western Australia during 1993 [291]. Nevertheless, CA-MRSA infections were only recognized as new public health threat when four healthy children died from sepsis and necrotizing pneumonia in the USA in 1998 [292].

CA-MRSA infections are defined by Center for Disease Control and Prevention (CDC), as “any MRSA infection diagnosed for an outpatient or within 48 hours of hospital admission”. Moreover, the patients should not have a medical history of MRSA infection, colonization or residence in a long-term care facility during the past year and should not carry indwelling catheters or other medical invasive devices (http://www.cdc.gov/mrsa/diagnosis/index.html). In spite of CDC definition being widely accepted, several other definitions of CA-MRSA have been published. Most of them are based on the timing of the MRSA isolation relatively to the time of admission, with or without evaluation of healthcare-associated risk factors; or by the absence of risk factors for HA-MRSA [293], [294].

CA-MRSA is highly transmitted by direct contact (e.g. skin-to-skin or contaminated fomites). CDC developed a conceptual model of CA-MRSA transmission that suggests that MRSA infection results from the following risks: crowding, cleanliness, compromised skin integrity, contaminated objects and prior antibiotic use [295]. Specific population groups seems to be at a higher risk for CA-MRSA infections, namely children and young people, injecting and intranasal drug users, people of low socioeconomic status, residents of inner city neighbourhoods, athletes, Native American and Pacific Islander populations, prisoners and soldiers, men who have sex with men, HIV patients, veterinarians, livestock handlers, and pet owners with median ages of 23 years old [87], [294]. Although skin and soft tissue infections (SSTIs) are the most common clinical manifestations of CA-MRSA infections, it can cause more serious infections as endocarditis, sepsis, necrotizing pneumonia, necrotizing

fasciitis, osteomyelitis, septic arthritis, folliculitis, cellulitis, impetigo, pyomyositis and myositis [87].

CA-MRSA isolates show specific phenotypic and genotypic features producing infections epidemiologically and clinically distinct from HA-MRSA. Phenotype characteristics of CA-MRSA include: faster grown rate than HA-MRSA strains, non-multidrug resistance profile (resistance to less than 3 classes of antibiotics in addition to beta-lactams) and lower MIC values for oxacillin (≤32mg/L) or imipenem (≤1mg/L) when compared with HA-MRSA (oxacillin: 128mg/L or imipenem: 32 mg/L) [296], [297]. In addition, CA-MRSA isolates carry smaller cassettes usually SCC*mec* types IV, V and VII [275], [298]. The linkage of CA-MRSA to smaller SCC*mec* cassettes seems to confer some fitness advantage in the community environment [296].

CA-MRSA has been strongly associated with enhanced virulence, when compared to HA-MRSA. The presence of CA-MRSA specific virulence factors and the high expression of the traditional MRSA virulence factors confer a higher pathogenic potential to CA-MRSA isolates [86], [299]. PVL is one of the virulence factors that have been epidemiological linked to CA-MRSA virulence and associated to recurrent and severe skin infections and necrotizing pneumonia [299], [300]. However, it has been reported that PVL genes alone are not responsible for the dissemination of CA-MRSA and also that PVL is not a reliable marker for CA-MRSA [91], [301]. Besides PVL, another element has been enrolled to an increased epidemicity of CA-MRSA – the mobile genetic element ACME [112]. This element confers a selective advantage and enhanced transmissibility [111]. Moreover, the virulence potential of CA-MRSA

has also been associated with enhanced expression of core-genome-encoded virulence determinants, such as alpha-toxin and phenol-soluble modulins (PSMs) [82], [90], [299], [302].

The origin of CA-MRSA is not quite understood. Some studies supporting two hypotheses: (1) if SCC*mec* has been acquired by different MSSA epidemic clones in the community or (2) if CA-MRSA is derived from HA-MRSA that escaped to the community [296], [303], [304]. In the beginning of CA-MRSA era, distinct genetic background of successful PVL-positive clones was associated to a continent specific geographic location (see Table 5) [305], [306].

Table 5: Molecular characteristics and geographic distribution of the predominant CA-MRSA clones (adapted from [275]–[277].

CA-MRSA clone	CC	ST	SCC*mec*	*spa* type	Geographic spread
USA300, PVL+/ACME+	8	8	IVa	t008, t024, t064, t190, t206, t211	Aus, Bel, Bul, Can, Cro, Cze, Den, Est, Fin, Fra, Gab, Ger, Hun, Ice, Ind, Isr, Ita, Jap, Jor, Leb, Net, Nor, NZ, Pol, SA, SR, Spa, Swe, Swi, UK, USA
USA400, PVL+	1	1	IVa	t127, t128, t174, t175, t176, t386, t558	Aus, Bel, Chn, Cro, Cyp, Den, Fin, Fra, Ger, Ice, Ids, Lat, Leb, Net, Nor, NZ, Rom, SA, Spa, Swe, Swi, Tai, UK, USA
Southwest Pacific, PVL+ (USA1100)	30	30	IVc	t012, t018, t019, t021, t138, t268, t276, t318, t338, t391	Aus, Bel, Can, Cyp, Cze, Den, Fin, Fra, Ger, Lat, Leb, Ice, Jor, Ita, Net, Nor, NZ, Pol, Spa, Swe, Swi, UK, USA
Taiwan, PVL+ (USA1000)	59	59	IVa/V/VII	t199, t216, t444	Aus, Bel, Cze, Den, Fra, Ger, Ice, Net, Nor, NZ, Pol, Spa, Swe, Swi, UK, USA
European, PVL+	80	80	IVc	t044, t131, t376, t416, t436, t455, t1109	Aus, Bel, Bul, Cro, Cyp, Cze, Den, Fin, Fra, Ger, Jor, Leb, Hun, Ice, Ita, Net, Nor, Spa, Swe, Swi, UK

Aus, Austria; Bel, Belgium; Bul, Bulgaria; Can, Canada; CHn, China; Cro, Croatia; Cyp, Cyprus; Cze, Czech Republic; Den, Denmark; Est, Estonia; Fin, Finland; Fra, France; Gab, Gabon; Ger, Germany; Hun, Hungary; Ice, Iceland; Ind, India; Ids, Indonesia; Isr, Israel; Ita, Italy; Lat, Latvia; Leb, Lebanon; Jap, Japan; Jor, Jordan; Net, Netherlands; Nor, Norway; NZ, New Zealand; Pol, Poland; SA, South Africa; Spa, Spain; Swe, Sweden; Swi, Switzerland; Tai, Taiwan; UK, United Kingdom; Uru, Uruguay; USA, United States of America

CA-MRSA infection was first reported in Western Australia in 1993 from indigenous peoples living in remote areas. Genotypic characterization revealed that these isolates belonged to ST30-IVc/PVL+ clone, so-called Southwest Pacific clone. Nowadays this clone that emerged as a new descent of the early CC30 phage type 80/81 clone (MSSA, PVL positive, penicillin resistant), is widely disseminated around the world [266], [307].

A few years later (1997-1999), in the USA, was described four fatal cases among children with CA-MRSA. These CA-MRSA isolates belonged to the clone USA400 clone (CC1, ST1-IVa, and PVL+) [11], [292]. Afterwards, the USA300 clone (CC8, ST8-IVa and PVL+) emerged and completely replaced the USA400 clone [308], [309]. USA300 is the predominant cause of STTIs in the community in the USA where it became endemic. This clone has been introduced into the American hospitals environment, displacing the classical HA-MRSA clones, becoming an important public-health problem [295], [310], [311]. Nowadays, USA300 clone have disseminated worldwide [312], [313].

In Asia, the first description of CA-MRSA was associated to the Taiwan clone (ST59-IVa/V_T and PVL+) [314], [315]. In contrast to the typical CA-MRSA antimicrobial susceptibility profile, the Taiwan clone was described as MDR [314], [316]. Currently, this clone remains the predominant clone circulating in Taiwan and has also been isolated in the USA (USA1000) and in Hong Kong [317], [318].

In Europe, the first CA-MRSA outbreaks were reported in 2003 among Greek strains collected in 1993 and 1998 to 2000 and were due to the existence of European clone (ST80-IVc/V) [319]. Since then, several reports of infection

associated with this clone were registered in different European countries, in North Africa and in the Middle East [306], [320]–[322]. CA-MRSA in Europe seems to be much more diverse than CA-MRSA in USA. Besides USA300 clone, other clones such as the European, the Taiwan and the Southwest Pacific clone are causes of infection in the community environment [323]. Nowadays, there is none reasonable explanation for this wide clonal type's variety in Europe.

In Spain, despite the high rates of HA-MRSA, the incidence of CA-MRSA seems to be low based on a small number of studies. The first description of a PVL-positive CA-MRSA was in 2003 and was related to the USA300 clone [324]. In a recent study published by Vindel and colleagues, a significant increase in CA-MRSA incidence from 2004 (0.25%) to 2012 (8.8%) was reported. Moreover, most of CA-MRSA clones circulating now in Spain were ST8-IVc and USA300 and resistance to other antimicrobials in addition to beta-lactams was not frequent (phenotypic characteristic of CA-MRSA) [325]. As previously referred, colonization by CA-MRSA has been found to be markedly increased in HIV-patients in the USA [326]. However, low prevalence (1-2%) of CA-MRSA colonization in HIV-infected patients in Barcelona (Spain) was detected [327].

Noteworthy, several reports suggest that the epidemiology of CA-MRSA have changed during the last years. Besides, the dissemination of the five main CA-MRSA clones (see Table 5), the emergence of CA-MRSA infections among hospitalized patients with risk factors for MRSA infection resulting in the emergence of MDR CA-MRSA, in the identification of variants of the prototypes clones or even in the emergence of new and less epidemic clones [287], [312],

[313]. Due to these changes in MRSA epidemiology, a new definition of CA-MRSA is needed. As already proposed, the best definition is the one based in a combination of phenotypic, genotypic and epidemiological data [328], [329].

3.4. Livestock-associated MRSA

MRSA of CC398 so-called livestock-associated MRSA (LA-MRSA), emerged as a new clonal lineage infecting or colonizing humans worldwide [330]–[333]. It has been suggested that a human MSSA CC398 lineage jumped and spread to livestock host, where it acquired the SCC*mec* cassette and *tet*(M) gene encoding methicillin and tetracycline resistance (Tet-R) respectively [80]. On the other hand, the LA-CC398 clone typically lacks proteins that are involved in human-specific innate immune system such as the staphylococcal complement inhibitor, encoded by the *scn* gene, harboured in the immune evasion cluster (IEC) region. LA-CC398 clustered into two major distinct phylogenetic clades: a basal human clade and a more derived livestock clade [79], [80].The absence of *tet*(M) gene is associated with the human-adapted clade and the loss of the IEC genes harbouring *scn* gene is associated with the livestock-adapted clade [80]. Recently, a new model of adaptation of the *S. aureus* CC398 lineage to diverse host has been suggested. This model proposed an ancestral prophage-free colonizing CC398 MSSA isolates that diverged into three subpopulations in parallel following the acquisition of mobile genetic elements (MGEs) (see Figure 10). Their findings highlighted the emergence of a novel subpopulation of CC398 isolates, responsible for invasive infections in various animals, with a considerable potential to colonize and infect humans, probably greater than that of human-adapted CC398 isolates that lack several clinically important *S. aureus*-associated virulence factors. This great

virulence capacity of this new subpopulation could be due to two genetic events: (i) the acquisition of Φ3 prophage carrying the IEC (*chp*, *sak*, amd *scn* genes) that facilitated animal-to-human spread and (ii) the acquisition of ΦMR11-like prophage and the MGEs encoding putative virulence genes that may significantly alter bacterial pathogenicity [334].

Some microbiologic traits define isolates belonging to the MRSA-ST398 clone: chromosomal DNA cannot be restricted by *Sma*I enzyme and CC398 strains generally present resistance to tetracycline, which is commonly used in pig farming [241], [335], [336]. Additionally, zinc and others metals are frequently used in animal feed formulations and may co-select for MRSA CC398 strains that carry the *czrC* zinc resistance gene, as suggested previously [337]. This hypothesis is supported by the fact that the vast majority of MRSA CC398 strains carry SCC*mec* type Vc, which contains the *czrC* gene. SCC*mec* type IV or VII like could also be identified in MRSA-CC398 isolates but in a lower frequency than SCC*mec* type V [80]. Moreover, it has been reported that most isolates of this LA-MRSA clone showed *spa* types t011, t034 and t108 [338]. Several studies reported that close animal contact increased the risk of being colonized or infected with MRSA-CC398 [243], [339], [340]. However, discordant findings were revealed about human persistent colonization and transmission routes between human and animals [341], [342]. MRSA strains with ST398 are mostly associated to skin and soft tissue infections (SSTIs) [343]. Although it has been suggested that MRSA-ST398 is less virulent than other human MRSA clones, due to the lack of important toxins such as Panton-Valentine leukocidin (PVL) and other enterotoxins, severe invasive infections

such as pneumonia, bacteraemia and endocarditis have been reported in Europe, Asia, and the USA [241], [244], [330]–[332], [344]–[346].

Transmission of MRSA-ST398 has also been reported in a nosocomial outbreak in the Netherlands [245].

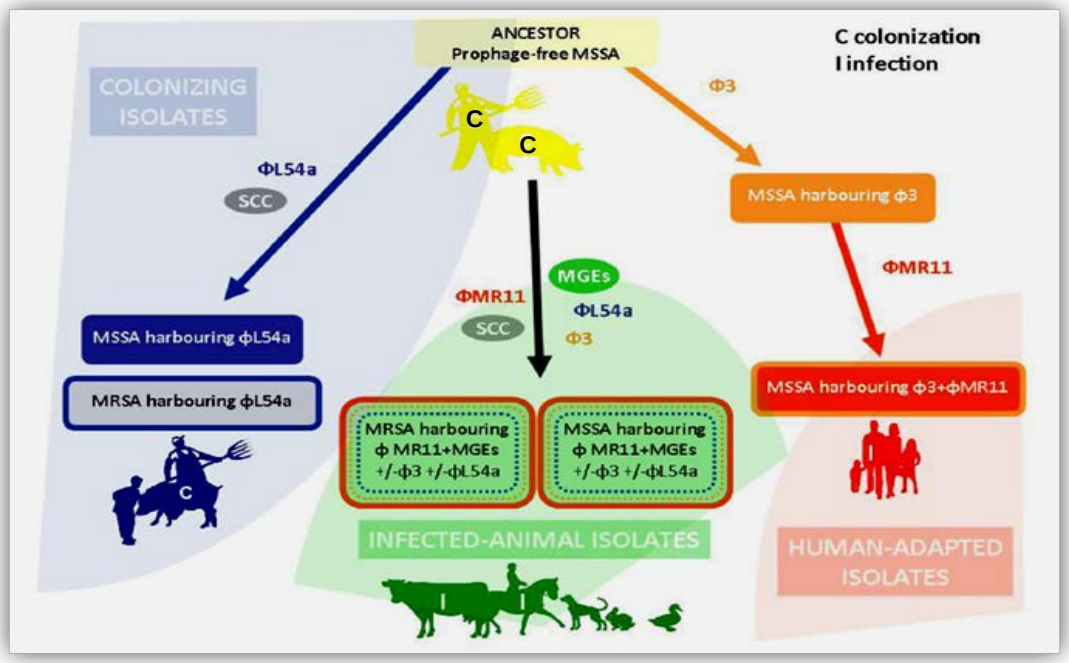


Figure 10: Schematic representation of the diversification within the CC398 lineage. Representatives of the ancestral prophage-free CC398 population were identified in the form of pig-borne colonizing isolates (upper part). The acquisition by such prophage-free isolates of the Φ3 prophage and the ΦL54a prophage resulted in the ancestral MSSA isolates: human isolates carrying the Φ3-prophage and pig-borne colonizing isolates carrying the ΦL54a prophage, respectively. The acquisition of the ΦMR11-like prophage by the human isolates resulted in human-adapted MSSA carrying Φ3- and ΦMR11-like-prophages; the acquisition by the pig-borne colonizing isolates of the SCC*mec* cassette resulted in the pig-bone colonizing MRSA isolates. Te acquisition of MGEs, including the ΦMR11-like prophage, which contains genes contributing to bacterial virulence, resulted in the emergence of MSSA and MRSA isolates responsible for infections in both livestock and pet species (lower part) (taken from [334]).

First descriptions of human colonization by MRSA-ST398 were reported in the Netherlands and France in mid 2000s [339], [346]. Since then, this clone has been reported in several European countries, but most cases were located in Belgium, Denmark, Austria and the Netherlands [330]. In Spain, MRSA-ST398 was first reported in Spain in 2010 associated to a skin lesion [347]. Remarkably, high rates of this clonal type among MRSA clinical isolates have only been detected in certain areas with higher density of pig farming i.e. Spain 5%, Netherlands 11.9% or Belgium 4.7% [80], [325], [330], [343].

4. Typing and proteomic methods used to characterize *Staphylococcus aureus*

Because the monitoring and control of *S. aureus*, and in particular MRSA, are of specific medical concern, several phenotypic and genotypic methods have been developed in the past 50 years. However, during the last years, phenotypic methods [e.g. biotyping, antimicrobial susceptibility testing, phage typing, whole-cell protein electrophoresis, zymotyping, multilocus enzyme electrophoresis (MLEE), capsule typing and serotyping] have been gradually replaced by molecular typing techniques [348], [349]. These molecular methodologies applied to epidemiological studies have been shown to have more ability to establish the prevalent and endemic clones versus sporadic and emerging *S. aureus* clones in a given community, country or geographic region. Molecular typing methods can be categorized in “band-based” and “sequence-based” methods. Variations in nucleotide sequence are detected indirectly by primer-binding and/or restriction sites [e.g. Pulsed-Field Gel Electrophoresis (PFGE)], using the molecular band-based methods. In contrast, the “sequence-based” method, determine the precise order of nucleotides and evaluate the DNA sequence variations [e.g. Multilocus Sequence Typing (MLST), *S. aureus* protein A (*spa*) typing, Amplified Fragment Length Polymorphism (AFLP), Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA), Random Amplified Polymorphic DNA (RAPD)]. The main advantages of the sequence-based molecular typing techniques over the band-based methods, are the highly reproducibility and interchangeability of data obtained [350]. The decision of the most appropriate typing strategy is highly dependent on the studying setting and purpose [351].

Overall, a good typing method should assess a marker that remains stable over time, have a high typeability, high discriminatory power, high degree of reproducibility, easy to perform and to interpret, generate interchangeable data, be inexpensive and not time consuming [352]. However, no single *S. aureus* typing method is capable of providing fully reliable information within the range of discrimination needed for public health intervention measures [351]. A strategy based on the combination of different typing methods for the molecular characterization of *S. aureus* is needed. This includes the characterization of isolates by PFGE, MLST, *spa* typing, *agr* typing, SCC*mec* typing (in the case of MRSA) and microarray technology [254], [353], [354]. More recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been used to discriminate different *S. aureus* lineages, therefore has become a valuable first-line tool for inexpensive and rapid typing of *S. aureus* in infection control [355]–[357]. Since these methodologies have been extensively used in the present work, a detailed description of each one will be provided.

4.1. Pulsed-field gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was first described in 1984 as tool for analyzing chromosomal DNA of eukaryotic organisms and afterwards has proven to be a highly effective molecular technique for several organisms including *S. aureus* [358], [359]. The method is based on the restriction of the chromosomal DNA by a restriction enzyme with relatively few recognition sites; in the case of *S. aureus* the most frequently used enzyme is the *Sma*I. After restriction of total DNA, around 10 to 30 fragments are generated that range

from 10 to 800Kb in length. These DNA fragments are resolved as a macro restriction pattern of distinct bands in a special electrophoresis apparatus, in which the electric field is applied in pulses that alternate in orientation, allowing the separation of the DNA fragments that range from 10 to 800Kb in length [358], [360].

To interpret the DNA fragment patterns generated by PFGE and transform them into epidemiologically useful information, consensus guidelines were created, aid understanding how to compare PFGE patterns and how random genetic events (e.g. point mutations, insertions, deletions of DNA) can alter the patterns. According to these PFGE guidelines, an isolate is considered to be closely related to the outbreak strain if its DNA restriction pattern produced by PFGE differs from the outbreak pattern by changes consistent with a single genetic event, i.e., a point mutation or an insertion or deletion of DNA, resulting in two or three band differences; possibly related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with two independent genetic events (i.e., four to six band differences); and unrelated to the outbreak strain if its restriction pattern differs from the outbreak by changes consistent with three or more independent genetic events (generally seven or more band differences) [359]. Besides visual interpretation, the resultant bands patterns analysis can be performed automatically using diverse bioinfomatic tools such as Bionumerics (Applied Maths Sint-Martens-Latem, Belgium), BioImage (BioImage Corp., Ann Arbor, MI), GelCompar (Applied Maths, Sint-Martens-Latem, Belgium) and Molecular Analyst Fingerprinting Plus (Bio-Rad, Hercules, California, USA).

PFGE is one of the most discriminatory and reproducible methods for *S. aureus* typing, being considered the “gold standard” method to investigate MRSA nosocomial outbreaks and to characterize the local and worldwide spread of *S. aureus* clones [275], [361]. However is technically demanding and time consuming. Moreover, data interpretation is complex, not reproducible, making it difficult to standardize and to exchange between laboratories, although several efforts have been made to harmonize the procedures and improve data interchangeability [361]–[364].

4.2. Multilocus sequence typing

Multilocus sequence typing (MLST) is a technique originally developed in 1998 for the characterization of *Neisseria meningitidis* lineages and was then extended to a variety of other microorganisms including *S. aureus* [365], [366]. The method is based on the nucleotide sequence analysis internal fragments (450-500bp) of seven housekeeping genes coding for essential functions. For *S. aureus*, this technique targets the following housekeeping genes: *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqiL* (acetyl coenzyme A acetyltransferase) [367].

The sequence of these genes can be submitted to a free online database (http://saureus.mlst.net/) where to each different gene sequence a distinct allele number is assigned and to each allelic profile, a sequence type (ST) is attributed (MLST profile 1-4-1-4-12-1-54 defined as ST125). The genetic relatedness between isolates can be obtained by analysis and comparison of MLST data using the eBURST (electronic based upon related sequence types)

algorithm (http://saureus.mlst.net/eburst) [151], [368]. This web tool is also freely available and allows the clustering of closely related STs into clonal complexes (CC), where the founder ST is the ST that became the most predominant and has diversified (by point mutation or recombination) producing variants that differ in only one or up to seven loci that are called single-, double-, or triple-locus variants (SLV, DLV and TLV). Furthermore, large clonal complexes typically contain subgroups hence, they have both primary and subgroup founders, where an SLV of the primary founder increases in frequency and diversified to generate a number of its own SLVs, therefore becoming a subgroup founder [368]. Regarding *S. aureus*, a CC could be defined as a cluster of isolates that share at least five identical loci [369]. The eBURST analysis can be represented graphically (Figure 11), where each ST is represented as a circle and the frequency of each ST (i.e., the number of isolates of the ST in the input data) is indicated by the area of the circle. The primary founder is given in blue, while subgroup founders are given in yellow. eBURST also allows all of the input data to be treated as a single group by selecting a group definition of zero of seven shared alleles. This procedure allows the clustering patterns among all isolates within a complete MLST database to be visualized as a single eBURST diagram named “population snapshot” (see Figure 11) [368]. Recently, a similar tool, the goeBURST (global optimal eBURST) that it is freely available at http://goeburst.phyloviz.net, was developed as an improvement of eBURST, with combined analysis of multiple data sources and using a different algorithm (Kruskal algorithm) solving the problem of finding the optimal forest based [370].

MLST has the big advantage of being suitable for long-term-surveillance as well as population evolutionary studies since it is based on variations in housekeeping genes that evolve slowly [367], [368]. Moreover, MLST generates accurate and reproducible data, due to the internationally standardized nomenclature submitted in a curated and updated database. However, this technique has moderate discriminatory power for epidemiological typing and it is time consuming, labourious and costly for use as a *S. aureus* primary typing tool [351].

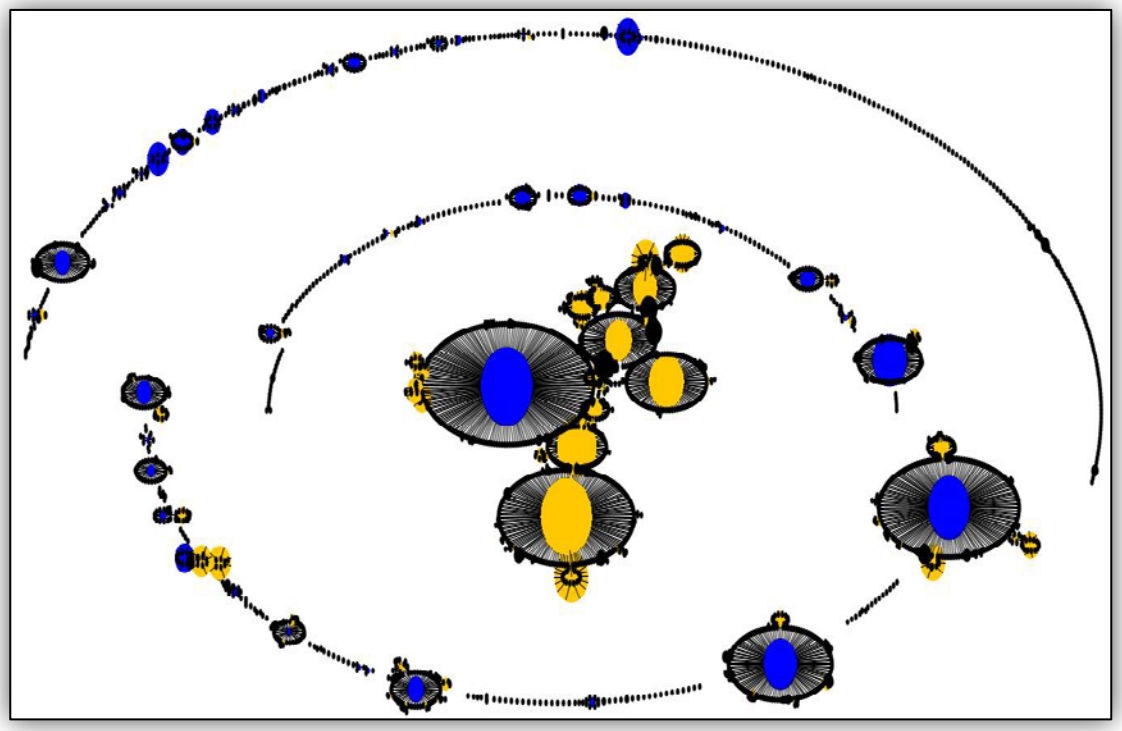


Figure 11: The *S. aureus* MLST population structure defined by eBURST. eBURST diagram representing a 'population snapshot' showing the clusters of linked STs and unlinked STs in the whole *S. aureus* MLST database (5630 isolates). Primary founders (blue) are positioned centrally in the cluster, and subgroup founders are shown in yellow. The dot size indicates the relative abundance of that particular ST in comparison to the other STs in the data set. ST labels have been removed (http://eburst.mlst.net ; accessed on August 22, 2015).

4.3. *S. aureus* protein A typing

S. aureus protein A (*spa*) typing was developed in 1996 by Frenay *et al*. to type S. aureus isolates, which is based on the sequencing of the polymorphic X region of the gene encoding the surface protein A (*spa*) [371]. The polymorphic X region consists of a variable number of short sequence repeats (SSR), usually of 24bp (although 21, 27 and 30bp repeats also described) located in the 3' coding region of the C-terminal cell wall attachment sequence of protein A [372]. This X region is flanked by well conserved sequences allowing the annealing of primers and the direct amplification and sequencing of the entire region [373], [374]. The high diversity in the X region of the gene is due to point mutations, deletions, duplications and insertions of repetitive units [375].

To the sequence of each different repeat an alphanumerical code is assigned and the combination of these specific repeats defines the *spa* type [376]. The nomenclature system more widely used uses the software package, Staphtype (Ridom GmbH, Würzburg, Germany) for automatic analysis and type attribution [376], [377]. It is a valuable tool for the comparison of *spa* typing data using a RIDOM nomenclature while providing as excellent quality control of data [378]. All *spa* sequence chromatograms are analysed and further synchronized to an online database, the SpaServer (http://spaserver2.ridom.de/index.shtml), implemented by the European SeqNet network (http://www.seqnet.org) that includes 60 laboratories from 29 European countries [350], [379]. Furthermore, the phylogenetic relatedness between different *spa* types can be established using the algorithm based upon repeat pattern (BURP) in association with StaphType software, which defines clusters of spa types into CC [380]. Several studies demonstrated a good concordance with other typing methods, namely

MLST CC generated by eBURST and PFGE types [323], [361], [377], [381]–[383].

Overall, *spa* typing has become one of the most useful typing methods for *S. aureus*, mainly due to its high reproducibility, tipeability, cost effectiveness, less time performance and easy data exchangeability [350], [378]. Despite being a single-locus typing method (low discriminatory power), *spa* typing have the capacity to cover genetic variations that accumulate micro- and macrovariations, and therefore may be used for outbreaks, local and global epidemiology, and population-based studies [377].

4.4. Accessory gene regulator typing

The accessory gene regulator (*agr*) quorum-sensing and signal transduction system was initially described in *S. aureus* as an element controlling the expression of virulence-associated protein genes [384], [385]. The *agr* locus consists of two-signal transduction components (AgrC as the signal receptor and AgrA as the response regulator) which drive the synthesis of RNA III, the principal effector of the *agr* response [386]. This operon is auto-induced by a peptide named AIP (autoinducing peptide), encoded within *agrD*, and located in the same locus. AIP diffuses into the target cell and acts as a receptor of the signal when the cell density is sufficient. This is a population density sensor or quorum sensing and is the only quorum-sensing system in the staphylococcal genome [38]. *S. aureus* isolates can be classified into four predominant groups according to the intra-variability of different regions of the *agr* operon. Isolates of the same group can activate the *agr* response in other isolates of the same group but they can usually inhibit those belonging to another group,

representing a novel form of bacterial interference [51]. It has been suggested that these *agr* groups may influence in the ability of *S. aureus* to colonize the host competing with other bacteria, including other staphylococci [51], [387]. *agr* polymorphism effects in the host are also likely to affect the host-to-host transmission and epidemiology of *S. aureus* clones [388]. Moreover, *agr* expression seems to be important during the early stages of infection [38].

Finally, *agr* specificity groups are usually identified by PCR amplification of the hypervariable domain of the *agr* locus using primers specific for each of the four major specificity groups (see Figure 12) [389].

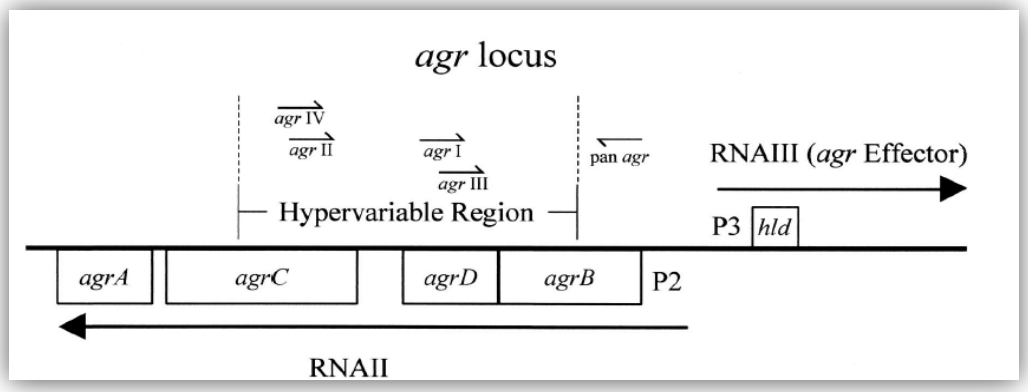


Figure 12: Schematic map of the *S. aureus agr* locus showing the locations of the different primers used for amplification of the hypervariable region (taken from [389]).

4.5. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing

The SCC*mec* typing is essential for MRSA clonal characterization, which is presently defined by the combination of MLST and SCC*mec* type [151], [259]. Since 2002, several SCC*mec* typing strategies have been described, to determine the structure of the different SCC*mec* types harboured by MRSA,

which are mostly PCR-based methods that, either by conventional PCR or multiplex PCR, rely on the amplification of different loci, where SCC*mec* elements are classified into types and subtypes [390]. Basically, SCC*mec* types are defined based on the combination of the type of *ccr* gene complex and the class of the *mec* gene complex, whereas SCC*mec* subtypes are determined based on the structural differences in J regions of each SCC*mec* type [172], [173], [177], [180], [296], [390]–[393].

Oliveira *et al*. described the first multiplex PCR strategy that detects SCC*mec* types I to IV [390]. An improved update of this multiplex PCR method was later published by Milheriço *et al*., where the inference of SCC*mec* types I to IV was optimized and two additional SCC*mec* type V and VI were included [180]. This new approach method is based on the sequencing of an internal fragment of the *ccrB* locus [394], [395]. Afterwards, the sequences can be submitted and analysed in an online database (www.ccrbtyping.net), where a *ccr*B allele is assigned. *ccr*B typing could be used as a promising SCC*mec* typing strategy since there is robust correlation among *ccr*B allelic clusters and SCC*mec* types [396].

Moreover, due to the increasing number of CA-MRSA infections, the typing and subtyping of SCC*mec* type IV became important. A multiplex PCR assay was developed to detect the variations within the J1 region, allowing the identification of SCC*mec* types IVa to IVh [180].

Besides PCR-based methods, real-time PCR assays have also been described for SCC*mec* typing. The most significant advantages of these methods are the short time and less labour in the PCR preparation and also the

easy manner to interpret results. However, these techniques require special equipment and reagents [397].

A recent report of the IWG-SCC (International Working Group on the Staphylococcal Cassette Chromosome elements) (http://www.sccmec.org.Pages/SCC_HomeEN.html), proposed some rules for the classification, nomenclature and definition of new SCC*mec* types, in order to avoid the ambiguities and inconsistencies in the published literature. These guidelines were established based on the type of *ccr* gene complex and class of *mec* gene complex in alternative to the former Roman numbers (i.e. type I (1B), indicating a SCC*mec* harbouring a type 1 *ccr* gene complex and a class B *mec* gene complex) [168].

The continuous emergence of new SCC*mec* structures and variants, like the recently described SCC*mec* types IX, X and XI, requires a permanent update of the existing strategies or the design of new ones [160], [398].

4.6. Microarray technology

A DNA microarray is a collection of DNA probes arranged in a solid surface, allowing the simultaneous detection of a high number of molecular targets [399], [400]. This technology have been widely used in the evaluation of genetic variation of *S. aureus* lineages, characterization of community and hospital MRSA clones and detection of resistance, virulence and epidemicity markers [400]–[404]. Depending on the number of probes we can differentiate low-density (hundred of probes) and high-density (hundreds of thousands of probes) DNA microarrays.

Generally, a microarray-based assay starts with a DNA extraction from the pathogen of interest. Afterwards, DNA is labelled either chemically or by an enzymatic reaction and hybridised to probes on the microarray. Unbound target DNA is removed during subsequent washing steps of different stringency. Successful hybridization is visualized and measured automatically by a scanner. Finally, analysis using dedicated software to assess the bacterial diversity is needed. The results retrieved from array technology are variable and depend on the customised array [399].

Because of the high costs of array assays, this approach has been used for research projects rather than in clinical routine laboratories, and sequence-based typing methods like MLST or *spa* typing have usually preferred [400].

The Alere StaphyType DNA microarray (Alere Technologies GmbH, Jena, Germany) consists of individual DNA microarrays mounted in 8-well microtiter strips, which detect 334 target sequences, including approximately 170 distinct genes and their allelic variants, covering species markers, markers for the recognition of SCC*mec*, and capsule types as well as *agr* groups, resistance genes, exotoxins, MSCRAMM genes, and others. The arrays are scanned on the designating reader and assignment of *S. aureus* isolates to clonal lineages is done automatically by software based on hybridization profiles [95]. The main advantages of this technique are the total cost that is much lower than that of MLST and can be performed within two working days. Nevertheless, microarray analysis is probably not the best method to type highly clonal species based on single-nucleotide polymorphisms (SNPs) and not allow the identification of sequences, which are not included in the array [399].

4.7. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

Recently, several studies report the ability of matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) provide a rapid and accurate identification of major human pathogens to the species level including bacteria, fungi and viruses [405]–[408]. Due to its accuracy and speed in species identification, the number of diagnostic laboratories that introduced this technique has been increasing in worldwide [409]–[411].

In general, identification of bacteria by MALDI-TOF MS using intact-cell method first involves the colony selection. Next, you can apply directly onto the MALDI test plate with formic acid or perform a full extraction with ethanol and formic acid and then added to the MALDI plate. In the third step, samples are then overlaid with matrix (α-cyano-4-hydroxycinnamic acid) and allowed to dry at room temperature. The plate is subsequently loaded into the MALDI-TOF MS instrument and the sample is bombarded by a short pulse of laser light. This bombardment results in the sublimation and ionization of both the sample and matrix. These liberated ions are separated based on their mass-to-charge ratio via a TOF tube, and a spectral representation of these ions is generated and analysed by the MS software, generating an MS profile. This profile is subsequently compared to a database of reference MS spectra and matched to either identical or the most related spectra contained in the database, generating and identification for bacteria contained within the sample [412].

Different approaches to this technique have been published in the last years. MALDI-TOF could be suitable for subspecies typing as exemplified for *Acinetobacter spp*., *Salmonella enterica*, *Listeria monocytogenes*, and *Streptococcus pneumoniae* [413]–[416].

MALDI-TOF MS technology has been able to discriminate between methicillin resistant and susceptible *S. aureus*, to discriminate MRSA clones or clonal lineages, to detect *S. aureus* delta-toxin production or to identify an outbreak of a PVL-positive MRSA strain, using several MALDI-TOF MS protocols and analyses [355]–[357], [417]–[420].

The majority of genetic typing methods are time consuming and expensive. MALDI-TOF MS fingerprinting could significantly improve MRSA surveillance by allowing routine real-time typing. Wolters *et al*. described a preliminary MALDI-TOF-based MRSA typing scheme including 13 specifics peaks that allowed the discrimination of 5 MRSA CC (CC5, CC8, CC22, CC30 and CC45) [355]. However, this strategy had several and important limitations that makes difficult to implement in routine analysis such as: no criteria for peak selection, manual selection peaks, tedious binary conversion and generation of hierarchical clusters for CC designation. Another strategy to differentiate the main *S. aureus* clonal lineages was evaluated by Josten *et al* [356]. In this work, the peak shifts of 6 *S. aureus* CC (CC5, CC22, CC8, CC45, CC30 and CC1) were correlated to point mutations, i.e., the genotypes of the strains. Besides the laborious analysis that needs to manually identify the biomarkers, this study could not identify a single marker peak to be specific for all isolates from distinct clonal groups [421].

Future studies should address the implementation of MALDI-TOF MS technique for automated discrimination of different MRSA clonal lineages and evaluation of interlaboratory reproducibility.

Chapter II

Objectives

1. To study the microbiological features of endemic methicillin-resistant *S. aureus* (MRSA) isolated from patients admitted to Hospital Universitari de Bellvitge over 25 years (1990-2014).

1.1. Analyse methicillin resistance evolution among *S. aureus* strains and study of MRSA's antimicrobial susceptibility to old and new anti-staphylococcal therapeutic agents.

1.2. Describe the molecular epidemiology of emergent and endemic MRSA clones using genotypic and proteomic-based methods.

1.3. Investigate the resistance and virulence genetic determinants of MRSA bacteremic clones.

2. To provide a phenotypic and genotypic analysis of the MRSA bacteremic isolates from patients admitted to Spanish hospitals from 2008 to 2009.

2.1. Study the rate of methicillin-resistance among *S. aureus* isolated in the hospitals involved in the multicenter study. Evaluation of the antibiotic resistance phenotypes among MRSA isolates.

2.2. Analyse the molecular characteristics of MRSA clones causing bloodstream infections using genotypic techniques.

Chapter III

Material and methods

1. Bacterial isolates

1.1 Hospital setting

Hospital Universitari de Bellvitge (HUB) is a 700-bed tertiary teaching hospital located in the Barcelona southern metropolitan area. The hospital belongs to the Institut Català de la Salut, the main public health supplier in Catalonia, Spain. The HUB is a reference centre for adult patients with approximately 30,316 admissions per year, serving a population of 1,000,000 inhabitants in both urban and rural areas.

1.2 Isolate collections

For the elaboration of this PhD thesis, three distinct collections of MRSA were studied.

Collection 1

This collection included 5061 MRSA clinical isolates that were recovered from single patients admitted to HUB between 1990 and 2014. Strains isolated from bacteremic patients admitted to the hospital in 1996 were not available to re-culture, so they could not be included in the molecular studies. Isolates were studied to analyse changes in the epidemiology of endemic MRSA observed in the HUB over the last 25 years (included in **objective 1**).

Collection 2

This collection included 207 MRSA resistant to tetracycline (clinical samples, n=138; active surveillance culture samples, n= 69) that were isolated between 2000 and 2013, accounting 7% of all MRSA recovered during this

period. Isolates were studied to evaluate the prevalence and molecular characteristics of MRSA-ST398 isolated in the HUB (2000-2013), a livestock-associated lineage that generally present resistance to tetracycline, which is commonly used in pig farming (included in **objective 1**).

Collection 3

This collection included 626 MRSA isolates from 616 bacteremic patients that were prospectively followed throughout a multicentre project in 23 Spanish hospitals (see Table 6), which are members of the Spanish Network for Research in Infectious Diseases (REIPI). Isolates were studied to provide a genotypic and phenotypic analysis of the MRSA bacteremic isolates from patients admitted in Spanish hospitals from June-2008 to December-2009 (included in **objective 2**). For each sample, a questionnaire assessing demographic and susceptibility testing data was filled in by the microbiologist of each hospital and delivered with the sample to our laboratory (see **Annex III**).

Table 6: Hospitals enrolled in this Spanish multicentre project and number of samples recovered by each setting between June-2008 to December-2009.

Hospital	Autonomous community	No. of samples
Hospital Universitari de Bellvitge	Cataluña	36
Hospital Universitari Vall d' Hebrón	Cataluña	55
Hospital Universitari Mútua de Terrassa	Cataluña	10
Hospital Universitari del Mar	Cataluña	19
Hospital Clinic	Cataluña	36
Hospital Parc Taulí	Cataluña	9
Hospital Arnau de Vilanova	C. Valenciana	9
Hospital Universitari Joan XXIII	Cataluña	17
Hospital Universitari Son Dureta	Islas Baleares	38
Hospital Universitari Gregório Marañon	Madrid	82
Hospital Universitari Ramón y Cajal	Madrid	60
Hospital Universitari 12 de Octubre	Madrid	34
Hospital Universitari Virgen del Rocío	Andalucía	33
Hospital Universitari Virgen Macarena	Andalucía	15
Hospital Reina Sofía	Andalucía	14
Hospital Universitari La Fe	Valenciana	11
Hospital Donostia	País Vasco	25
Hospital Marques de Valdecilla	Cantabria	26
Hospital Gral. Yagüe	Castilla y León	12
Hospital de Cruces	País Vasco	26
Hospital San Pau	Cataluña	25
Hospital San Pedro de La Rioja	La Rioja	31
Hospital de Terrassa	Cataluña	3
Total No of samples		626

1.3 Sample processing and storage

Collections 1 and 2

All isolates were previously identified as MRSA by HUB microbiology laboratory and conserved in Tryptic Soy Broth (TSB, Becton Dickinson™, Heidelberg, Germany) with 15% glycerol at -80ºC. These original cultures were inoculated on Columbia agar with 5% sheep blood (COS, Becton Dickinson™, Heidelberg, Germany) and incubated at 37ºC for at least 18 hours, in order to obtained isolated colonies and confirmed the purity of the stored samples. If the culture was pure, a single colony was selected and plated again on COS at 37ºC for 18 hours. If the cultured presented colonies with distinct morphologies, each type of colony was streaked on COS until a pure culture was obtained and tested for clumping factor and protein A detection by the latex slide agglutination test (Pastorex Staph-plus; Bio-Rad Laboratories, Madrid, Spain) and DNase production (DNase E-test Agar, bioMérieux, Marcy l'Étoile, France). The purified bacterial cultures were conserved in TSB with 15% glycerol at -80ºC as a duplicate and used in this thesis for further analysis.

Collection 3

The swabs (Deltalab S.L.U., Spain) received from each hospital were inoculated on COS (COS, Becton Dickinson™, Heidelberg, Germany) and incubated at 37ºC for at least 18 hours. If the culture was pure, a single colony was selected and plated again on COS at 37ºC for 18 hours. If the cultured presented colonies with distinct morphologies, each type of colony was streaked on COS until a pure culture was obtained. All *S. aureus* isolates were

identified by latex agglutination (Pastorex Staph-plus; Bio-Rad Laboratories, Madrid, Spain) and DNase production (DNase E-test Agar, bioMérieux, Marcy l'Étoile, France). The purified bacterial cultures were conserved in TSB with 15% glycerol at -80ºC and used for further analysis.

2. Phenotypic characterization

2.1 Antibiotic susceptibility testing

Disk-diffusion method

All MRSA isolates from all isolate collections were tested for antimicrobial susceptibility through Kirby-Bauer disk-diffusion method according to the Clinical Laboratory Standard Institute (CLSI) guidelines from 2012 [202]. The antimicrobial agents tested were: Penicillin (P), Oxacillin (OX), Cefoxitin (FOX), Erythromycin (E), Clindamycin (CC), Trimethoprim-sulphamethoxazole (SXT), Gentamicin (GM), Tobramycin (NN), Tetracycline (TE), Rifampicin (RA), Ciprofloxacin (CIP), Chloramphenicol (C), Quinupristin-dalfopristin (SYN), Teicoplanin (TEC) and Linezolid (LZD).

Bacterial suspensions were prepared in TSB and adjusted to a turbidity of 0.5 McFarland ($1x10^8$ CFU/mL). The bacterial suspensions were inoculated on Mueller-Hinton II agar (MH2, bioMérieux, Marcy l'Étoile, France) and incubated at 37ºC for 24 hours with discs impregnated with a known antibiotic concentration (BBL™, Sensi-Disc™, Becton Dickinson™, Heidelberg, Germany). The halo formed around the disc was measured (in mm) and the isolates were considered susceptible (S), intermediate resistant (I) and resistant

(R) according to CLSI guidelines (see Table 7). Control strain was *S. aureus* ATCC 25923.

Table 7: Clinical breakpoints for *S. aureus* according to CLSI 2012 guidelines [202].

Antibiotic	Disk content	Halo diameter (mm)			MIC (µg/mL)		
		S	I	R	S	I	R
P	10 units	≥29	-	≤28	≤0.12	-	≥0.25
OX	1µg	13	11-12	≤10	≤2	-	≥4
FOX	30µg	≥22	-	≤21	≤4	-	≥8
E	15µg	≥23	14-22	≤13	≤0.5	1-4	≥8
CC	2µg	≥21	15-20	≤14	≤0.5	1-2	≥4
SXT	1.25/23.75µg	≥16	11-15	≤10	≤2/38	-	≥4/76
GM	10µg	≥15	13-14	≤12	≤4	8	≥16
NN	10µg	≥15	13-14	≤12	≤4	8	≥16
TE	30µg	≥19	15-18	≤14	≤4	8	≥16
RA	5µg	≥20	17-19	≤16	≤1	2	≥4
CIP	5µg	≥21	16-20	≤15	≤1	2	≥4
C	30µg	≥18	13-17	≤12	≤8	16	≥32
SYN	15µg	≥19	16-18	≤15	≤1	2	≥4
VA	-	-	-	-	≤2	4-8	≥16
TEC	30 µg	≥14	11-13	≤10	≤8	16	≥32
DA	-	-	-	-	≤1	-	-
LZD	30µg	≥21	-	≤20	≤4	-	≥8

Microdilution method

Minimum inhibitory concentration (MIC) was determined in all MRSA blood isolates by the microdilution method by using commercial panels (ESTEN1F, Sensititre, Izasa, Barcelona, Spain) (see Figure 13). Each panel was inoculated with 50µl of cation-supplemented Mueller-Hinton broth containing an inoculum density of approximately $1x10^8$ CFU/mL and was carried out with the Sensititre autoinoculator. Following 24h (overnight) of incubation at 37°C, visual determination of the MIC was made by recording the lowest concentration of each drug in a dilution series that inhibited observable growth. The results were interpreted following the CLSI guidelines for the same antimicrobial agents that

were analysed on disc-diffusion method (see Table 7) [202]. Control strain was *S. aureus* ATCC 29213.

In this PhD thesis, *S. aureus* isolates were considered multidrug-resistant if they were resistant to three or more antimicrobial classes, in addition to beta-lactams.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PEN 8	AMP 8	VAN 16	TEI 16	ERY 4	CLI 2	OXA+ 2	FUS 16	DAP 8	GEN 8	LZD 4	NIT 64
B	PEN 4	AMP 4	VAN 8	TEI 8	ERY 2	CLI 1	OXA+ 1	FUS 2	DAP 4	GEN 4	LZD 2	NIT 32
C	PEN 2	AMP 0.5	VAN 4	TEI 5	ERY 1	CLI 0.5	OXA+ 0.5	FUS 1	DAP 2	GEN 2	RIF 2	SYN 2
D	PEN 0.25	AMP 0.25	VAN 3	TEI 4	ERY 0.5	CLI 0.25	OXA+ 0.25	TOB 8	DAP 1	GEN 1	RIF 1	SYN 1
E	PEN 0.12	AMP 0.12	VAN 2	TEI 2	ERY 0.25	TET 8	CIP 2	TOB 4	SXT 4/76	GEN 128	RIF 0.06	NEG CTRL
F	PEN 0.03	MUP 256	VAN 1.5	VAN 0.5	TYG 1	TET 4	CIP 1	TOB 2	SXT 2/38	GEN 500	FOS+ 128	POS CTRL
G	IMI 8	MUP 2	VAN 1	VAN 0.38	TYG 0.5	TET 1	CIP 0.5	TOB 1	SXT 0.03/0.59	AUG 8/4	FOS+ 64	POS CTRL
H	IMI 4	MUP 1	VAN 0.75	VAN 0.25	TYG 0.25	DT1	DT2	FOXS	STR 1000	AUG 4/2	FOS+ 32	POS CTRL

Figure 13: ESTEN1F (Sensititre^{TM}) 96-well panel template used for MIC determinations.

PEN, Penicillin; IMI, Imipenem; AMP, Ampicillin; VAN, Vancomycin; TEI, Teicoplanin, ERY, Erythromycin, TYG, Tigecycline; CLI, Clindamycin; TET, Tetracycline; DT1 and DT2, D-test MLS induction phenotype; OXA, Oxacillin, CIP, Ciprofloxacin, FUS, Fusidic acid; TOB, Tobramycin; FOXS, Cefoxitin screening; DAP, Daptomycin; SXT, Trimethoprim-sulphamethoxazole; STR, Streptomycin; GEN, Gentamicin; AUG, Amoxicillin-clavulanic acid; LZD, Linezolid; RIF, Rifampicin; FOS, Fosfomycin; NIT, Nitrofurantoin; SYN, Quinupristin-dalfopristin; NEG CTRL and POS CTRL, Negative and positive growth controls.

Vancomycin, teicoplanin and daptomycin MICs were also studied in both collections by E-test (AB bioMérieux, Solna, Sweden). The bacterial suspensions (turbidity of 0.5 on the McFarland scale) were inoculated in MH2

agar and incubated at 37ºC for 24 hours. The results were interpreted following the CLSI guidelines (2012) (Table 7) [202].

2.2 Evaluation of vancomycin and daptomycin activity

Vancomycin and daptomycin activity was investigated using 96-well commercial microdilution plates (Sensititre™, Cat. No. EUNOVD, Trek Diagnostics, West Sussex, England) in a selected collection of blood isolates (**collection 1**; **objective 1**; **studies 2** and **3**). Vancomycin and daptomycin were tested at concentrations 0.06 to 64 and 0.015 to 32 mg/L, respectively (see Figure 14). In addition to the appropriate concentration of daptomycin, the wells also contain approximately 25mg/L calcium. *S. aureus* ATCC 29213 was included each day of testing.

Isolate Number		1	2	3	4	5	6	7	8	9	10	11	12
1	A	DAP 0.015	DAP 0.03	DAP 0.06	DAP 0.12	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16	DAP 32
	B	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 64	POS CON
2	C	DAP 0.015	DAP 0.03	DAP 0.06	DAP 0.12	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16	DAP 32
	D	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 64	POS CON
3	E	DAP 0.015	DAP 0.03	DAP 0.06	DAP 0.12	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16	DAP 32
	F	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 64	POS CON
ATCC 29213	G	DAP 0.015	DAP 0.03	DAP 0.06	DAP 0.12	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16	DAP 32
	H	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	VAN 64	POS CON

Figure 14: EUNOVD (Sensititre™) 96-well panel template used for vancomycin and daptomycin MIC determination.

VAN, Vancomycin; DAP, Daptomycin; POS CON, Positive control

Microdilution procedure

Inoculum were prepared from 24 hours bacterial culture on Tryptic Soy Agar with 5% sheep blood (TSA, bioMérieux, Marcy l'Étoile, France) and some isolates colonies from these plates were inoculated into a 5mL tube of sterile deionized water (Cat. No. T3339, Trek Diagnostics, West Sussex, England) and the turbidity was adjusted to 0.5 McFarland standard. This suspension was transferred (50µl) to 11mL cation adjusted Mueller Hinton broth tube (CAMHB, Cat. No. T3462, Trek Diagnostics, West Sussex, England) and each well plate was inoculated with 100µL of this organism suspension. Therefore, the total calcium concentration in the daptomycin wells inoculated with CAMHB is approximately 50mg/L.

Inoculum purity was verified by spread 10µL of inoculated broth from the growth control well on a TSA plate and incubates at 35ºC for 24 hours. The MIC test was repeated if colonies other than the test strain are present.

Inoculum concentration was verified by transfer 10µL from the positive control well into a 5mL tube of sterile saline (1:500 dilution) and spread (100µL) to a TSA plate (1:10 dilution). Colonies were counted after 24h of incubation at 35ºC (each colony representing 5,000 colony-forming unit (CFU) in original broth). A colony count range of 60-140 is equivalent to the optimal 3-7x10^5 CFU/mL.

Each microdilution plate was sealed with an adhesive plate seal and then were incubated at 35ºC for 24 hours before examination for MIC values. Visual

determination of the MIC was made by recording the lowest drug concentration showing no visible bacterial growth.

Minimal bactericidal concentration (MBC) procedure

MBC values were determined by plating the entire contents (100µL) of the MIC well, and wells at four dilutions above the MIC onto individual TSA plates. The plates were incubated 48 hours at 35ºC and colonies counted from the plate of the highest concentration well that exhibits growth.

CFU rejection value was calculated as follows:

Initial inoculum (CFU/mL) x volume (mL) plated (0.1mL) x allowable viable percent (0.001) = CFU rejection value

- A plate showing > the CFU rejection value does not meet the 99.9% endpoint.
- A plate showing ≤ the CFU rejection value indicate ≥ 99.9% killing.
- The MBC is the lowest concentration that is bactericidal to 99.9% of the original inoculum.

Vancomycin tolerance was defined by MBC/MIC ≥32.

2.3 Delta-haemolysin production assay

Delta-haemolysin production was measured by cross-streaking a selected collection of blood isolates (**collection 1**; **objective 1**; **study 3**), perpendicularly to RN4220 (prototype strain), which produces only β-haemolysin on a COS plate [422]. The plates were incubated 24h at 35ºC and 24h plus at 4ºC. This test can usually identify the three staphylococcal haemolysins active on COS

plates – α, β and δ – because of the interactions between them: β-haemolysin enhances lysis by δ-haemolysin, but inhibits lysis by α-haemolysin. A functional *agr* locus (delta-haemolysin production), can be identified by the presence of synergistic haemolysin within the β-haemolysin zone produced by RN4220.

3. Molecular characterization

3.1 DNA extraction

All isolates that were characterized by PCR-based methods were submitted to a chromosomal DNA extraction. Isolates were grown overnight on COS at 37ºC. Three up to five colonies were resuspended in 100µL of ST buffer (TrisHCl 6mM; NaCl 1M; EDTA 0.1M, pH8; sodium deoxycholate 0.2%; sarcosyl 0.5%), with 1µL of lysozyme 50mg/ml (Sigma-Aldrich, St. Louis, USA) and 2.5µL of lysostaphin 10 mg/ml (Sigma-Aldrich, St. Louis, USA). These suspensions were incubated at 30 minutes for 37ºC and 5 minutes at 95ºC. After mixing, this suspension was further diluted 1:100 in water. The DNA was subsequently stored at -20ºC.

3.2 Pulsed-field gel electrophoresis

The protocol used for pulsed-field gel electrophoresis (PFGE) analysis was previously described by Chung *et al*. with some modifications [362]. Briefly, isolates were previously plated on COS and incubated at 37ºC overnight. One microliter inoculation loop were resuspended in 200µL of PIV (0.01mM Tris-HCl, pH8; 1 M NaCl). A 1:200 dilution of the suspension was prepared and the optical density (OD) (620nm) was measured on the spectrophotometer

(Utrospec 2000, Pharmacia Biotech). PIV was added to each bacterial suspension in order to obtain a final OD of 5.0. The agarose discs were prepared by doing a mixture of 1:1 of the bacterial suspensions (OD=5) with 1.5% (w/v) low melting agarose (Ultrapure™ LMP Agarose, Invitrogen, USA). Twenty microliter droplets of this mixture were deposited onto a parafilm-coated glass and covered with a microscope slide to form a disc-like shape. The discs were incubated at -20ºC for 5 minutes and left at room temperature for 10 minutes. Lysis of cells was performed by putting the discs in 1mL of ST buffer, 50µg/mL RNase A (Sigma-Aldrich, St. Louis, USA), 100µg/mL lysozyme and 50µg/mL lysostaphin and incubated at 37ºC for at least 5 hours followed by decantation of this solution and replacement by Proteinase K solution (1mg/mL) [0.5M EDTA (pH9); 1% sarcosil; 1mg proteinase K (Sigma-Aldrich, St. Louis, USA)]. This solution was left overnight at 50ºC. The discs were washed five times in 1X TE [10mM Tris-HCl (pH8); 1mM EDTA (pH8)], each time for at least 30 minutes at room temperature, and stored at 4ºC in this solution until use.

Total DNA contained in the agarose discs was restricted with 10U of *Sma*I enzyme (New England Biolabs Inc., Beverly, MA, USA) in 40 µL of 1X NEB4 buffer and 0.8µL of BSA (200µg/mL). Nontypable MRSA strains by *Sma*I were further restricted with 10U of *Apa*I enzyme (New England Biolabs Inc., Beverly, MA, USA) in 40 µL of 1X NEB4 buffer and 0.8µL of BSA (200µg/mL). In both cases, discs were incubated at 25ºC for 6 hours.

The chromosomal DNA fragments were then separated in a 1% agarose gel (Certified™ Megabase Agarose, Bio-Rad, Hercules, California, USA) in 0.5X TBE (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8). The electrophoresis

was carried out in a CHEF-DRIII apparatus (Bio-Rad, Hercules, California, USA) with 0.5X TBE as running buffer. The Lambda Ladder PFG Marker (New England Biolabs Inc., Beverly, MA, USA) was used as molecular weight marker. The running conditions were: 6V/cm with initial pulses of 1 second, which increased until 30 seconds, for a total run of 18 hours using *Sma*I enzyme and 6V/cm with pulse times from 0.5 to 15 seconds for a total run of 20 hours using *Apa*I enzyme. Next, the gel was stained with a 0.375 µg/mL ethidium bromide solution (Bio-Rad, Hercules, California, USA) for about 20 minutes followed by visualization and data recording under UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

Analysis of PFGE restriction patterns

The resulting restriction patterns were interpreted by visual inspection, using the criteria of van Belkum *et al*. [352]. To identify each PFGE type of **collections 1** and **2**, the alphanumerical nomenclature assigned by HUB microbiology laboratory before 2009, was continued during the last 6 years, in order to facilitate the analysis and interpretation of epidemiological data obtained over the last 25 years. PFGE types and subtypes of **collection 3** were assigned using a numerical nomenclature (i.e. PFGE 1.2, PFGE type 1 and subtype 2).

PFGE types were additionally defined in FINGERPRINTING^{TM} II software, version 3.0 (Bio-Rad Laboratories, Inc. Madrid, Spain) in **objective 1.2** (**study 6**) and **objective 2.2**. PFGE patterns were normalized using the lambda ladder marker and was included in two lanes of each gel. The similarity of PFGE

patterns within and between gels was performed with the following settings: optimization and position tolerance of 0.7% for **objective 1.2** (**study 6**, **article 2**); optimization was set at 0% and band position tolerance at 0.7% for **objective 1.2** (**study 6**, **article 3**) and optimization and band position tolerance were both set at 0.6% for **objective 2.2**. To define PFGE types the similarity coefficient cut-off was set 80%. A dendogram was generated by the unweighted-pair group method with arithmetic mean based on Dice coefficients. The groups defined at these thresholds shown to approximate those defined using visual inspection.

3.3 *S. aureus* protein A typing

Typing of the *S. aureus* protein A (*spa*) locus was carried by first amplifying the *spa* gene as described previously [376]. Representative isolates of each PFGE type and subtype were selected among isolates of all collections and then were characterized by this technique.

The amplification employed 10µM of *spa*-1113F (5'-TAA AGA CGA TCC TTC GGT GAG C-3') and 10µM of *spa*-1514R (5'-CAG CAG TAG TGC CGT TTG CTT-3'), in a 50µL mixture containing 37.55µL of sterile water (B.Braun Medical SA, Barcelona, Spain), 3µL of template DNA, 10mM dNTPs mix (Qiagen GmbH, Hilden, Germany), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA) and 1.25U of AmpliTaq DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted

of an initial denaturation (9 minutes 30 seconds at 95ºC) followed by 35 cycles of denaturation (30 seconds at 95ºC), annealing (30 seconds at 62ºC), and extension (1 minute at 72ºC), with a single final extension (10 minutes at 72ºC). The resulting DNA fragments were run in a 1% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 30 minutes and were visualized by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes.

The PCR products were visualized under UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA) and then were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). The purified PCR products were sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The software Ridom StaphType^{TM} v2.2.1 (Ridom GmbH, Munster, Germany) was used for *spa* sequence analysis. Each different repeat was identified by an alphanumerical code and the combination of these repeats was assigned a specific *spa* type. The BURP (Based Upon Repeat Pattern) algorithm (Ridom StaphType^{TM} software) was used to calculate spa clonal complexes (*spa*CC) with the defaults parameters for the group/cluster definition: “exclude *spa* types that are shorter than 5 repeats” and “*spa* types are clustered if cost is less or equal to 6”.

3.4 Multilocus sequence typing

Multilocus sequence typing (MLST) was performed as described by Enright *et al.* in representative isolates of each PFGE type and subtype of all isolate collections [367]. Seven PCR amplicons were obtained for each isolate using the 7 housekeeping genes primers (see Table 8). Each 50µL PCR mixture contained 36µL of sterile water (B.Braun Medical SA, Barcelona, Spain), 3µl of DNA template, 1.15µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 0.8µL of each forward and reverse primer (25µM), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), and 1.25U of AmpliTaq DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted of an initial denaturation (9 minutes 15 seconds at 95ºC) followed by 35 cycles of denaturation (45 seconds at 95ºC), annealing (45 seconds at 55ºC), and extension (1 minute at 72ºC), with a single final extension (10 minutes at 72ºC).

The PCR products (5µL) were run in a 1% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 30min. The bands were visualized by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA). The PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and then sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The sequences were analysed using the software SeqMan (v5.03, DNASTAR, Inc.). MLST types were assigned by submitting the sequences to the *S. aureus* database on the website (http://saureus.mlst.net) and eBURST algorithm (eBURST v.3) was applied to all MLST data in order to assign MLST clonal complexes (CC) (htpp://eburst.mlst.net).

The global optimal eBURST (goeBURST) algorithm implemented in PHYLOViZ (http://goeburst.phyloviz.net) was applied to our isolates **collection 3** and was used to visualise CCs, including both single and double locus variants. Moreover, this data was supplemented with isolate metadata including geographical origin [370], [423].

Table 8: Primers used for amplification of the seven housekeeping genes [367].

Gene	Primer	Sequence (5'-3')	Sequence length (bp)
Carbamate kinase (*arcC*)	*arcC*-Up	TTGATTCACCAGCGCGTATTGTC	456
	arcC-Dn	AGGTATCTGCTTCAATCAGCG	
Shikimate dehydrogenase (*aroE*)	*aroE*-Up	ATCGGAAATCCTATTTCACATTC	456
	aroE-Dn	GGTGTTGTATTAATAACGATATC	
Glycerol kinase (*glpF*)	*glpF*-Up	CTAGGAACTGCAATCTTAATCC	465
	glpF-Dn	TGGTAAAATCGCATGTCCAATTC	
Guanylate kinase (*gmk*)	*gmk*-Up	ATCGTTTTATCGGGACCATC	429
	gmk-Dn	TCATTAACTACAACGTAATCGTA	
Phosphate acetyltransferase (*pta*)	*pta*-Up	GTTAAAATCGTATTACCTGAAGG	474
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA	
Triosephosphate isomerase (*tpi*)	*tpi*-Up	TCGTTCATTCTGAACGTCGTGAA	402
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC	
Acetyl coenzyme A acetyltransferase (*yqiL*)	*ypiL*-Up	CAGCATACAGGACACCTATTGGC	516
	ypiL-Dn	CGTTGAGGAATCGATACTGGAAC	

3.5 Staphylococcal cassette chromosome *mec* (SCC*mec*) typing

SCC*mec* typing by multiplex PCR

SCC*mec* typing was performed for all MRSA isolates that were previously characterized by MLST. The determination of SCC*mec* structure was performed by multiplex PCR strategy previously described by Milheiriço *et al*. [180]. The following reference strains were used as controls: COL (SCC*mec* type I), N315 (SCC*mec* type II), ANS46 (SCC*mec* type III), MW2 (SCC*mec* type IVa), WIS (SCC*mec* type V) and HDE288 (SCC*mec* type VI).

Reaction mixture contained 6.7µL sterile water (B.Braun Medical SA, Barcelona, Spain), 5µL of DNA template, 0.8µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), 1.25U of AmpliTaq DNA polymerase (Applied Biosystems, Roche, New Jersey, USA), 0.2µM of primers kdp F1 and kdp R1; 0.4µM of primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1F, and SCC*mec* V J1R; 0.8µM of primers *mecI* P2, *mecI* P3, *dcs* F2, *dcs* R1, *mecA* P4, *mecA* P7, ccrB2 F2, *ccrB2* R2, *ccrC* F2, and *ccrC* R2 (see Table 9). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA), and thermal cycling reactions consisted of an initial denaturation (12 minutes at 95ºC) followed by 30 cycles of denaturation (30 seconds at 95ºC), annealing (1 minute at 48ºC), and extension (1 minute at 72ºC), with a single final extension (4 minutes at 72ºC).

The PCR products (10µL) were resolved in a 3% agarose gel (Certified™ Megabase Agarose, Bio-Rad, Hercules, California, USA) in 0.5X TBE (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) containing 0.1µg/mL of ethidium bromide, at 4V/cm for 2.5 hours (Bio-Rad, Hercules, California, USA) and were then visualized under UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

Table 9: Primers used in SCC*mec* multiplex PCR [180].

Primer name	Primer sequence (5'-3')	Primer specificity (SCC*mec* type, region)	Amplicon size (bp)
CIF2 F2	TTC GAG TTG CTG ATG AAG AAG G	I, J1 region	495
CIF2 R2	ATT TAC CAC AAG GAC TAC CAG C		
ccrC F2	GTA CTC GTT ACA ATG TTT GG	V, *ccr* complex	449
ccrC R2	ATA ATG GCT TCA TGC TTA CC		
RIF5 F10	TTC TTA AGT ACA CGC TGA ATC G	III, J3 region	414
RIF5 R13	GTC ACA GTA ATT CCA TCA ATG C		
SCCmec V J1 F	TTC TCC ATT CTT GTT CAT CC	V, J1 region	377
SCCmec V J1 R	AGA GAC TAC TGA CTT AAG TGG		
dcs F2	CAT CCT ATG ATA GCT TGG TC	I, II, IV, and VI, J3 region	342
dcs R1	CTA AAT CAT AGC CAT GAC CG		
ccrB2 F2	AGT TTC TCA GAA TTC GAA CG	II and IV, *ccr* complex	311
ccrB2 R2	CCG ATA TAG AAW GGG TTA GC		
kdp F1	AAT CAT CTG CCA TTG GTG ATG C	II, J1 region	284
kdp R1	CGA ATG AAG TGA AAG AAA GTG G		
SCCmec III J1 F	CAT TTG TGA AAC ACA GTA CG	III, J1 region	243
SCCmec III J1 R	GTT ATT GAG ACT CCT AAA GC		
mecI P2	ATC AAG ACT TGC ATT CAG GC	II and III, *mec* complex	209
mecI P3	GCG GTT TCA ATT CAC TTG TC		
mecA P4	TCC AGA TTA CAA CTT CAC CAG G	Internal positive control	162
mecA P7	CCA CTT CAT ATC TTG TAA CG		

ccrB typing

SCC*mec* type assignments were confirmed by *ccrB* typing in a collection of blood isolates, including representatives of different PFGE and *spa* types (**collection 1**; **objective 1**; **study 5**), as previously described by Oliveira *et al*. [395], [396]. *ccrB* typing was carried by sequencing the *ccrB* fragment amplified

by PCR. The primer sequences were as follows: ccrB F1, CGW YTR GCW MGW AAY ACH TC; and *ccrB* R1, CTT TTC GWC KYT TWT CRY TCC. The predicted amplicon sizes was 496bp. PCR was performed in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes; amplifications in 35 cycles (denaturation at 94ºC for 30 seconds, annealing at 42ºC for 1 minute and extension at 72ºC for 2 minutes) and a final extension at 72ºC for 4 minutes. In each reaction (final volume of 50µL), 5µL of template DNA, 1X AmpliTaq PCR buffer with 1.5mM $MgCl_2$ (Applied Biosystems, Roche, New Jersey, USA), 2mM dNTP mix (MBI Fermentas, Hanover, MD, USA), 100pmol of each primer and 1.25U AmpliTaq DNA polymerase (Applied Biosystems, Roche, New Jersey, USA).

The PCR products (2µL) were run in a 1% agarose gel (Seakem LE, Lonza, Rockland, ME, USA) using 1X TAE buffer (0.5M Tris; 0.05M EDTA, pH8; 1M acetic acid), containing 0.1 µg/mL ethidium bromide (Bio-Rad, Hercules, California, USA) at 5V/cm for one hour. The bands were visualized in an UV light in Gel-doc XR apparatus (Bio-Rad, Hercules, California, USA). The PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and then sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The sequences were analysed using the software SeqMan (v5.03, DNASTAR, Inc.). These *ccrB* internal sequences in the FASTA format were submitted in a typing tool that is freely available at http://www.ccrbtyping.net.

Based on this assignment, a prediction of *ccrAB* loci and SCC*mec* type is also outputted [396].

3.6 Accessory gene regulator typing

Accessory gene regulator *(agr)* typing was performed for all MRSA isolates that were previously characterized by MLST. The detection of *agr* polymorphisms was performed by multiplex PCR strategy with a set of 1 forward primer (*agr*1-4$_{Sa}$-1) and 4 reverse primers (*agr*1$_{Sa}$-2, *agr*2$_{Sa}$-2, *agr*3$_{Sa}$-2, *agr*4$_{Sa}$-2) as described previously (see Table 10) [353]. The following strains were used as controls of PCR amplification: RN6390 (*agr*I), RN6607 (*agr*II), RN8465 (*agr*III) and RN4850 (*agr*IV). Reaction mixture contained 29.5µL sterile water (B.Braun Medical SA, Barcelona, Spain), 5µL of DNA template, 2µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), 1.25U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, New Jersey, USA) and 25µM of each primer. The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) under the following conditions: an initial 9 minutes and 15 seconds denaturation step at 95ºC followed by 35 cycles (45 seconds of denaturation at 95ºC, 1 minute of annealing at 53ºC, and 1 minute of extension at 65ºC) and a final extension step at 65ºC for 10 minutes.

The PCR products (20µL) were resolved in a 2% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) in 0.5X TBE (890mM Tris; 890mM Boric acid; 20mM EDTA (pH8) and were visualized by ethidium bromide (0.375

µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

Table 10: Primers used for amplification of *agr* polymorphism [353].

Gene	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
***agr*$_{Sa}$**	*agr*1-4$_{Sa}$-1	ATG CAC ATG GTG CAC ATG C	
***agr*-1$_{Sa}$**	*agr*1$_{Sa}$-2	GTC ACA AGT ACT ATA AGC TGC GAT	439
***agr*-2$_{Sa}$**	*agr*2$_{Sa}$-2	TAT TAC TAA TTG AAA AGT GCC ATA GC	572
***agr*-3$_{Sa}$**	*agr*3$_{Sa}$-2	GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G	321
***agr*-4$_{Sa}$**	*agr*4$_{Sa}$-2	CGA TAA TGC CGT AAT ACC CG	657

3.7 Detection of Panton-Valentine leukocidin gene

The presence of Panton-Valentine leukocidin (PVL) genes, *lukS*-PV and *lukF*-PV, was investigated by PCR in selected isolates of all collections as described by [94]. The strain used for positive control of *pvl* gene was *S. aureus* MW2 and the primers were luk-PV-1 (5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3') and luk-PV-2 (5'-GCA TCA AST GTA TTG GAT AGC AAA AGC-3'). The expected size of the amplicon obtained with this protocol was 433bp. The amplification reaction contained 2µL of template DNA in a final volume of 50µL containing 33.55µL of sterile water (B.Braun Medical SA, Barcelona, Spain), 1.25µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 2µL of each forward and reverse primer (24pmol), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), and 1.25U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted

of an initial denaturation (12 minutes at 95ºC) followed by 30 cycles of denaturation (45 seconds at 95ºC), annealing (45 seconds at 55ºC), and extension (1 minute at 72ºC), with a single final extension (2 minutes at 72ºC).

The PCR products (16µL) were resolved by electrophoresis through 1% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 1 hour (Bio-Rad, Hercules, California, USA). This was followed by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

3.8 Detection of arginine catabolic element

The presence of arginine catabolic element (ACME) was investigated in selected isolates of all collections by a multiplex PCR assay that uses primer sets specific for the *arcA* gene and a 16SrRNA universal target sequence was performed. The 16SrRNA gene, common to all bacteria was used as an internal control to identify potential false-negative results. The primers used were *arcA* F (5'-GAG CCA GAA GTA CGC GAG-3´) and *arcA* R (5'-CAC GTA ACT TGC TAG AAC GAG-3') for *arcA* gene and *Rib*1 (5'-GGA ATT CAA A(T/G,1:1) GAA TTG ACG GGG GC-3') and *Rib*2 (5'-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC-3') for 16SrRNA gene [111], [424]. The expected size of the amplicon obtained with this protocol was 733bp and 479bp for *arcA* and 16SrRNA genes respectively. The strain used as positive control was a *S. aureus* USA300 strain. The reaction mixture was prepared in a final volume of 50µL as follows: 5µL of template DNA, 29.75µL of sterile water (B.Braun

Medical SA, Barcelona, Spain), 2µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 0.5µL of each forward and reverse primer (25µM), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), and 1.25U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted of an initial denaturation (12 minutes at 95ºC) followed by 35 cycles of denaturation (45 seconds at 95ºC), annealing (45 seconds at 56ºC), and extension (1 minute at 65ºC), with a single final extension (4 minutes at 65ºC).

The PCR products (15µL) were run in a 0.75% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 1 hour (Bio-Rad, Hercules, California, USA). This was followed by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

3.9 Detection of *cfr* (chloramphenicol-florfenicol resistance) gene

Isolates resistant to both clindamycin and chloramphenicol, potentially linezolid-resistant (**collection 1**; **objective 1**; **study 4**) were screened for the *cfr* presence. The presence of *cfr* gene was analysed by PCR and the expected size of the amplicon was 746bp [136]. Two strains carrying the *cfr* gene and previously characterized were used as controls [220]. The amplification reaction contained 3µL of template DNA in a final volume of 50µL containing 36µL of

sterile water (B.Braun Medical SA, Barcelona, Spain), 1.15µl dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 0.8µL of each forward (5'-TGA AGT ATA AAG CAG GTT GGG AGT CA-3') and reverse primer (5'-ACC ATA TAA TTG ACC ACA AGC AGC-3') (25µM), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), and 1.25U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted of an initial denaturation (12 minutes at 95ºC) followed by 35 cycles of denaturation (30 seconds at 95ºC), annealing (30 seconds at 55ºC), and extension (3.5 minutes at 72ºC), with a single final extension (10 minutes at 72ºC).

The PCR products (16µL) were resolved by electrophoresis through 1% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 45 min (Bio-Rad, Hercules, California, USA). This was followed by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA).

3.10 Detection of *rpoB* (RNA polymerase) gene

An internal sequence of gene *rpoB* of 432bp (nucleotides 1216 to 1648) was amplified by PCR and sequenced to detect the rifampicin resistance-associated mutations in a selected collection of blood isolates (**collection 1**;

objective 1; **study 3**) [425]. The oligonucleotide sequences used were *rpoB*-for (5'-GTC GTT TAC GTT CTG TAG GTG-3') and *rpoB*-rev (5'-TCA ACT TTA CGA TAT GGT GTT TC-3').

Amplification was carried out in a 50µL volume containing 36.15 µL of sterile water (B.Braun Medical SA, Barcelona, Spain), 20µM of each primer, 1µL of dNTPs 10mM (Qiagen GmbH, Hilden, Germany), 5µL of 10X PCR Buffer II (Applied Biosystems, Roche, New Jersey, USA), 3µL of $MgCl_2$ 25mM (Applied Biosystems, Roche, New Jersey, USA), and 1.25U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA) and thermal cycling reactions consisted of an initial denaturation (9 minutes and 30 seconds at 95ºC) followed by 35 cycles of denaturation (30 seconds at 95ºC), annealing (30 seconds at 62ºC), and extension (1 minute at 72ºC), with a single final extension (10 minutes at 72ºC).

The PCR products (5µL) were run in a 1% agarose gel (Agarosa Estandar Media, Ecogen, Barcelona, Spain) using 0.5X TBE buffer (890mM Tris; 890mM Boric acid; 20mM EDTA, pH8) at 5V/cm for 30min. The bands were visualized by ethidium bromide (0.375 µg/mL) (Bio-Rad, Hercules, California, USA) staining for 20 minutes in an UV light in Gel-doc 2000 apparatus (Bio-Rad, Hercules, California, USA). The PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), and then sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The sequences were analysed using the software SeqMan (v5.03, DNASTAR, Inc.). The nucleotide sequences obtained were compared to the *rpoB* wild type sequence from *S. aureus* subsp. *aureus* (GenBank accession number: X641172) using the clustalw software http://www.ebi.ac.uk/tools/clustalw/index.html. Rifampicin-susceptible strains used as controls were ATCC29213 (rifampicin and methicillin susceptible *S. aureus*) and ATCC700698 (rifampicin susceptible MRSA). Two representatives of the Iberian clone were used as rifampicin-resistant MRSA controls: ATCCBAA44 and PER88 [426], [427].

3.11 DNA microarray genotyping

Virulence genes and resistance encoding genes were characterized using the DNA microarray Identibac MRSA Array-Strip (Staphy-Type, Alere Technologies Ltd, Jena, Germany; stripe version). The DNA microarray technique was performed in a selected collection isolates (**collection 1**; **objective 1**; **studies 6** and **8**) and were utilized as described previously [354], [400], [428]. Briefly, the array covers 334 target sequences, which correspond to approximately 180 distinct genes and their allelic variants. Target genes, primers and probes were previously published in the electronic supplementary material of Monecke *et al* [304].

Preparation of genomic DNA

S. aureus isolates were cultured on COS agar (Becton Dickinson™, Heidelberg, Germany) (overnight at 37ºC). Two inoculation loops of colony material were suspended in a lysis buffer (Staphy-Type, Alere Technologies

Ltd, Jena, Germany; stripe version) containing lysostaphin, lysozyme, ribonuclease A, Tris-HCl, EDTA and Triton X-100. Culture material was incubated for 60 minutes at 37 °C and 550 rpm in a thermoshaker (Thermo shaker PHMP, Grant-bio, UK). After incubation, DNA preparation was processed according to the protocol of the DNeasy blood & tissue kit (Qiagen GmbH, Hilden, Germany).

Microarray-based genotyping

For amplification and labelling, a linear and thermally synchronized primer elongation reaction was performed using a PCR apparatus (Applied Biosystems 2720 Thermal Cycler, Applied Biosystems, CA, USA). This allowed all targets to be amplified and labelled simultaneously, using one specific primer for each target. The 10µL labelling reaction comprised a mixture of 5µL of sterile water (B.Braun Medical SA, Barcelona, Spain), 4.9µL of PCR buffer B1 (primers, dNTPs, biotin-16-dUTP), 0.1µL of Therminator polymerase and 0.5-2µg of target DNA. Amplification conditions comprised an initial denaturation (5min at 96ºC), followed by 45 cycles of 20 seconds at 50ºC, 30 seconds at 72ºC, and 20 seconds at 96ºC. Labelled amplicons from this reaction were used afterwards for hybridization.

Hybridization procedures were identical for both approaches, and have been described previously [304]. In brief, the labelled sample was denaturated, chilled on ice and hybridized to the array. This was followed by washing steps, and by the addition of a blocking reagent. Afterwards, 100µL of horseradish peroxidase substrate (Staphy-Type, Alere Technologies Ltd, Jena, Germany;

stripe version) were added per well and then incubated 6 minutes at 25ºC avoiding direct sunlight.

After the liquid being removed completely, the resulting array image was recorded and analysed using the ArrayMate reader (Staphy-Type, Alere Technologies Ltd, Jena, Germany; stripe version). The assignation of isolates to clonal complexes and/or to sequence types was determined by an automated comparison of hybridization profiles to previously MLST-typed reference strains in an ArrayMate 1.1 database [354], [400].

3.12 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS)-based typing.

MALDI-TOF/MS typing were performed to identify clonal complex (CC)-specific biomarker peaks in isolates (**collections 1 and 2**; **objective 1**; **study 7**) that were representative of the 4 major MRSA clonal CCs in our hospital.
All isolates studied by this tool were previously well-characterised by molecular typing techniques that have already been referred in this chapter such as PFGE, MLST, SCC*mec*, *spa* and *agr* typing.

Sample preparation and MALDI-TOF/MS data acquisition

Bacterial cultures were grown overnight on COS agar (Becton Dickinson™, Heidelberg, Germany) at 37ºC and subjected to ethanol-formic acid extraction according to [413].

MALDI-TOF/MS was conducted on a Microflex LT (Bruker Daltonics, Bremen, Germany) benchtop instrument controlled by the FlexControl software

(version 3.0; Bruker Daltonics). Spectra were acquired in linear positive mode at a laser frequency of 20Hz using the MALDI Biotyper (version 2.0; Bruker Daltonics, Bremen, Germany) pre-processing standard method and the Biotyper main spectrum (MSP) identification standard method (mass range: 2,000 to 20,000 Da). The spectra were externally calibrated using the Bruker Daltonics Bacterial Test Standard.

One microliter of each bacterial extract was spotted onto a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. For MALDI-TOF/MS analysis, each spotted sample was overlaid with 1µL of a saturated matrix solution (α-cyano-4-hydroxy-cinnamic acid; Bruker Daltonics, Bremen, Germany) in 50% acetonitrile-2.5% trifluoroacetic acid (Sigma-Aldrich chemical Co.) and air-dried. Acquired spectrum for each spot was the sum spectrum accumulated from 250 measurements (5x50 laser shots on different locations according to a predefined lattice raster).

Selected bacterial isolates were included within a reference (36 strains) or a validation set (46 strains). Selection was performed as to include as much diversity as possible within both sets, prioritizing the reference set whenever an equitable distribution was not possible. Selection criteria were based on MLST variation or on *spa*-typing whenever MLST was too homogeneous. Bacterial extracts of the reference and validation sets were spotted onto a MALDI target plate in 10 and 3 replicas respectively. Spots for the reference set were measured three times, resulting in 30 mass spectra for each isolate, and those

of the validation set were analysed twice, resulting in 6 mass spectra per isolate.

MALDI-TOF/MS data analysis

The resulting raw spectra for each isolate of the reference set were loaded into the ClinProTools software (version 2.2; Bruker Daltonics, Bremen, Germany) and grouped into 4 different classes, each class containing the spectra profiles of isolates belonging to the same CC, and used to calculate the average spectra for each class (CC). Spectra were prepared by recalibration, baseline subtraction (Top Hat with a 10% minimal baseline width), peak selection and average peak list calculation ranging from 2,000 to 10,000 mass to charge ratio values (*m/z)*. *m/z* values from the average spectra of each CC were extracted and informative peaks were identified according to their statistical significance, as determined by the different statistic tests supported by ClinProTools (Bruker Daltonics, Bremen, Germany): Anderson-Darling test, t-/ANOVA test and Wilcoxon/Krustal-Wallis test. Informative peaks were those showing a significant difference between the four CCs whether: the p value for the Anderson-Darling test was > 0.05 and for the t-/ANOVA or Wilcoxon/Krustal-Wallis test was ≤ 0.05 or if the p value for the Anderson-Darling test was ≤ 0.05 and for the Wilcoxon/Krustal-Wallis test was ≤ 0.05 [429].

Classification models were generated using the genetic algorithm (GA), supervised neural network (SNN), and QuickClassifier (QC) algorithms, in order to identify the algorithm providing the best recognition capability. For model preparation, null spectra exclusion and spectra grouping were enabled. Recalibration was performed with a 1,000 parts per million (ppm) maximal peak

shift and 30% match to calibrant peaks. Not recalibrated spectra were excluded. For model generation, the default settings were left unaltered. The recognition capability and cross validation values were calculated to demonstrate the reliability and accuracy of the model.

BioTyper MSPs representing each CC were created with the BioTyper MSP creation method using the following parameters: frequency threshold for spectra adjusting: 50; frequency threshold for score calculation: 5; maximal mass error of the raw spectrum: 2,000; desired mass tolerance of the adjusted spectrum: 500; furthermore accepted mass tolerance of a peak: 800; parameter of the intensity correction function: 0.25. Subtyping MSPs for each CC were derived from the typing MSPs using the BioTyper subtyping MSP creation method with default parameters. Specific weights for each peak in the subtyping MSPs were set to 0% or replaced by the weight value calculated with the pattern generation models for informative peaks.

Chapter IV

Results

1. Microbiological features of endemic methicillin-resistant *S. aureus* (MRSA) isolated from patients admitted to Hospital Universitari de Bellvitge (HUB) over 25 years (1990-2014).

Article 1: Sierra JM, **Camoez M,** Tubau F, Gasch O, Pujol M, Martin R, Domínguez MA. Low Prevalence of *cfr*-Mediated linezolid resistance among methicillin-resistant *Staphylococcus aureus* in a Spanish hospital: case report on linezolid resistance acquired during linezolid therapy. *PLoSOne*. 2013;8(3):e59215.

Article 2: **Camoez M,** Sierra JM, Pujol M, Hornero A, Martin R, Domínguez MA. Prevalence and molecular characterization of methicillin-resistant *Staphylococcus aureus* ST398 resistant to tetracycline at a Spanish hospital over 12 years. *PLosOne*. 2013;8(9):e72828.

Article 3: **Camoez M,** Càmara J, Tubau F, Hornero A, Pujol M, Liñares J, Domínguez MA. Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Barcelona, Spain, 2012-2013. *Eur J Clin Microbiol Infect Dis* (submitted).

Article 4: **Camoez M,** Sierra JM, Dominguez MA, Ferrer-Navarro M, Vila J, Roca I. Automated categorization of methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin Microbiol Infect*. 2015 Oct 16. pii: S1198-743X(15)00908-8. doi: 10.1016/j.cmi.2015.10.009.

1.1. Analysis of methicillin resistance evolution among *S. aureus* strains and study of MRSA's antimicrobial susceptibility to old and new anti-staphylococcal therapeutic agents.

The percentage of methicillin resistance among clinical *S. aureus* isolates obtained at the Microbiology Department of Hospital Universitari de Bellvitge (HUB) during the 1990-2014 period is shown in Figure 15. The MRSA average among *S. aureus* over the last 11 years (2004-2014) was stabilized around 24%.

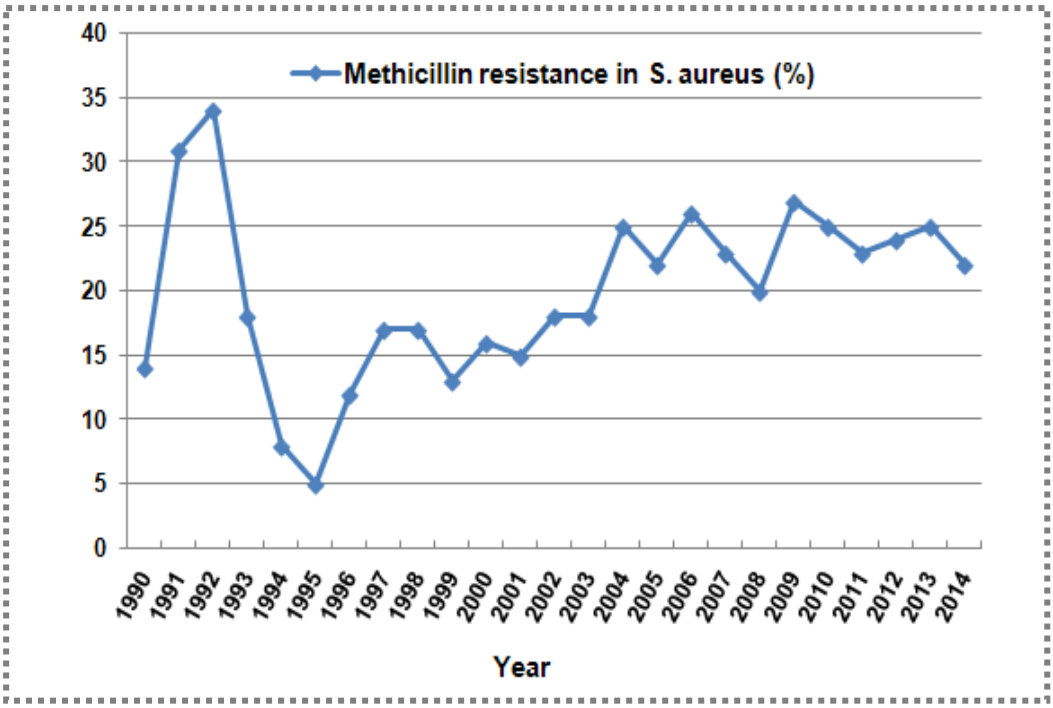


Figure 15: Evolution of methicillin resistance, among *S. aureus* strains isolated in the HUB during the 1990-2014 period.

Study 1: Antimicrobial susceptibility of the MRSA clinical strains isolated in the HUB (1990-2014).

In this study, 5061 clinical MRSA strains were isolated from patients admitted in the HUB from 1990 to 2014. Antibiotic susceptibility testing was performed in all isolates and the global resistance percentages of MRSA clinical isolates to antimicrobial agents is shown in Table 11. All the isolates were susceptible to vancomycin, and teicoplanin following EUCAST and CLSI breakpoints.

Table 11: Global resistance percentages of MRSA clinical isolates (n=5061) to antimicrobial agents.

	1st Period (1990-1994)	2nd Period (1995-1999)	3rd Period (2000-2004)	4th Period (2005-2009)	5th Period (2010-2014)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Isolates No.	1116	559	923	1184	1279
Resistance to antibiotics					
Ciprofloxacin	1049 (94)	542 (97)	877 (95)	1137 (96)	1151 (90)
Tobramycin	1049 (94)	559 (100)	794 (86)	900 (76)	780 (61)
Erythromycin	1049 (94)	475 (85)	655 (71)	900 (76)	768 (60)
Clindamycin	1049 (94)	475 (85)	406 (44)	533 (45)	281 (22)
Gentamicin	1049 (94)	442 (79)	231 (25)	462 (39)	166 (13)
Tetracycline	1049 (94)	369 (66)	111 (12)	36 (3)	64 (5)
Rifampicin	1049 (94)	369 (66)	166 (18)	367 (31)	51 (4)
Trimethoprim-sulfamethoxazole	22 (2)	28 (5)	37 (0.4)	83 (0.7)	26 (0.2)

Resistance to ciprofloxacin remained high over the whole period. Decreased in the rate of aminoglycosides and other antibiotics was the result of a clonal replacement observed during second and third periods (from 1995 to 2004) when the Iberian clone (resistant to all antibiotics in table 11, but cotrimoxazole) was replaced by other clones with variable resistance to amynoglicosides, rifampicin, erythromycin and clindamycin. Of note,

erythromycin resistance (MS_B phenotype), mediated by *msr*A gene was increasing over the years being found in the 38% of the strains during the fifth period.

The 5061 MRSA strains were grouped in 100 resistance patterns (RP), although, 75% (75/100) were detected after 2000. RPs that were frequently found among MRSA strains isolated in the HUB during the studied period are shown in Table 12.

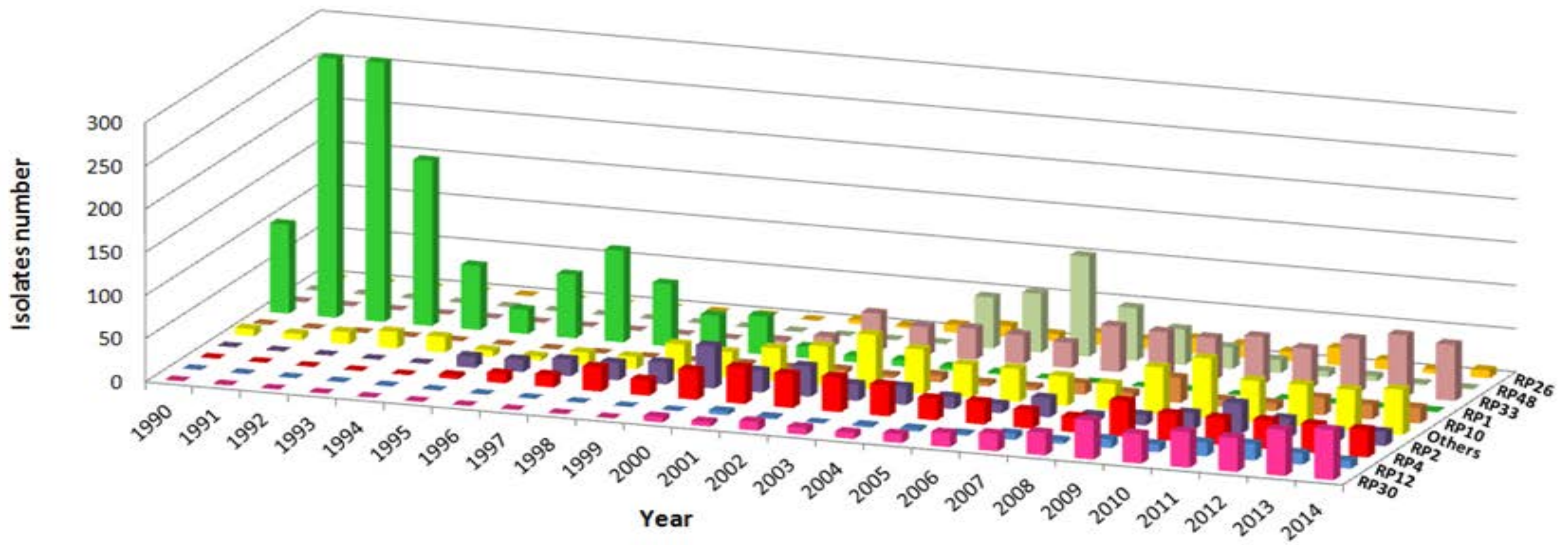
Table 12: Resistance patterns (RP) mostly found among MRSA strains isolated in HUB.

Code	Resistance pattern	Freq. (%)	Code	Resistance pattern	Freq. (%)
RP1	E, CC, G, NN, CIP, RA, TE	28.61	**RP27**	NN	0.34
RP2	E, CC, NN, CIP	8.10	**RP30**	CIP	7.49
RP3	E, CC, NN	0.06	**RP33**	E, NN, CIP	12.69
RP4	NN, CIP	11.01	**RP37**	E, CC	0.36
RP7	E, CC, G, NN, TE, SXT, CIP, RA	0.41	**RP38**	E, C, NN, CIP	2.09
RP10	E, CC, CIP	2.94	**RP42**	TE	0.40
RP12	only beta-lactams	1.98	**RP48**	E, CC, G, NN, CIP, RA	7.96
RP13	E, CC, C, NN, CIP	0.47	**RP49**	E, NN, TE, CIP	0.04
RP15	C, NN, CIP	0.32	**RP53**	E	0.10
RP17	E, CC, G, NN	0.10	**RP56**	NN, TE, CIP	0.10
RP18	E, C, G, NN, CIP	0.26	**RP61**	G, NN, RA, CIP	0.14
RP20	E, CC, G, NN, CIP	2.21	**RP62**	E, G, NN, TE, CIP	0.06
RP21	E, C, CIP	0.71	**RP65**	E, NN, CIP, RA	0.12
RP22	G, NN	0.12	**RP71**	TE, CIP	0.20
RP23	E, CC, C, G, NN, CIP	0.18	**RP74**	E, CC, NN, TE, CIP	0.08
RP24	C, NN	0.08	**RP90**	NN, TE	0.04
RP26	E, CIP	3.00	**Others**		7.23

E=Erythromycin; **CC**=Clindamycin; **NN**=Tobramycin; **CIP**=Ciprofloxacin; **G**=Gentamicin; **C**=Chloranphenicol; **TE**=Tetracycline; **RA**=Rifampicin; **SXT**=Trimethoprim-sulphamethoxazole

During the first decade (1990-1999), the dominant antibiotic RP among MRSA strains showed resistance to erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampicin and tetracycline. Isolates were only susceptible to vancomycin and co-trimoxazole. This resistance pattern, named RP1, was found in 82% of the MRSA isolates (1,369/1,675; 82%) and the isolates belonged to the Iberian clone. In the following years, this clone was

gradually replaced by isolates with variable RP and susceptible to more antibiotic classes (see Figure 16).



	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	Total n
RP30	0	0	0	0	0	0	0	0	0	0	6	5	10	8	7	10	16	20	26	45	34	41	39	53	58	378
RP12	0	0	0	0	0	0	0	0	0	0	0	4	2	0	2	3	2	7	4	10	9	16	19	13	9	100
RP4	0	0	0	0	0	4	11	14	28	18	34	42	39	40	36	25	26	21	18	41	31	31	33	33	32	557
RP2	0	0	0	0	0	13	14	19	20	25	49	25	35	19	21	14	10	22	7	12	19	35	22	13	16	410
Others	8	7	14	19	18	7	5	14	14	33	29	38	45	63	51	38	38	34	30	54	69	48	48	47	52	823
RP10	0	0	0	0	0	0	0	0	0	0	2	2	5	3	8	4	4	13	7	29	8	9	20	17	18	149
RP1	103	300	382	191	74	28	73	106	72	40	44	13	7	7	4	2	0	0	0	1	0	0	0	0	1	1448
RP33	0	0	0	0	0	0	0	0	0	0	3	12	44	33	36	33	29	53	51	49	55	46	62	71	65	642
RP48	0	0	0	0	0	0	0	0	0	0	0	0	0	1	59	68	116	61	41	25	15	7	6	2	1	402
RP26	0	0	0	0	0	0	0	0	0	1	0	5	3	9	13	8	14	11	17	15	11	20	11	5	9	152
Total n	111	307	396	210	92	52	103	153	134	117	167	146	190	183	237	205	255	242	201	281	251	253	260	254	261	5061

(RP30: ciprofloxacin; RP12: beta-lactams; RP4: tobramycin, ciprofloxacin; RP2: erythromycin, clindamycin, tobramycin, ciprofloxacin; RP10: erythromycin, clindamycin, ciprofloxacin; RP1: erythromycin, clindamycin, gentamicin, tobramycin, tetracycline, ciprofloxacin, rifampicin; RP33: erythromycin, tobramycin, ciprofloxacin; RP48: erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampicin; RP26: erythromycin, ciprofloxacin).

Figure 16: Antibiotic resistance patterns (RP) frequently found among MRSA strains isolated in the HUB during the 1990-2014 period.

Study 2: Comparative *in vitro* activity of daptomycin and vancomycin against MRSA blood strains isolated in the HUB.

Daptomycin (DA) is the first of the cyclic lipopeptides that shows rapid bactericidal activity against *S. aureus*, including strains tolerant or resistant to glycopeptides. This work aimed to assess the *in vitro* activity of DA compared to vancomycin (VA) against selected MRSA isolates from bacteremic adult patients attended at HUB.

Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values to DA and VA were performed by microdilution in 185 MRSA blood isolates. The strain selection included isolates from the 1990-2009 period and the following clones: CC5 (ST5, ST125, ST146-SCC*mec*IV-*agr*II) (n=76); Iberian ST247-SCC*mec*I-*agr*I (n=54); ST228-SCC*mec*I-*agr*I (n=27); EMRSA-15 ST22-SCC*mec*IV-*agr*I (n=13) and ST8-SCC*mec*IV-*agr*I (n=15). Analyses of MICs and MBCs variations throughout the studied period are shown in Tables 13 and 14.

Table 13: MICs (mg/L) results at various vancomycin (VA) and daptomycin (DA) concentrations during two different periods.

Period	Total nº of isolates	VA (mg/L)			MIC_{50}	MIC_{90}	Geometric mean	DA (mg/L)			MIC_{50}	MIC_{90}	Geometric mean
		≤0.5	1	2				≤0.5	1	2			
1990-1999	52	3	40	9	1	2	1.08	35	17	0	0.5	1	0.60
2000-2009	133	66	66	1	1	1	0.71	117	13	3	0.5	1	0.42
Total nº of isolates	185	69	106	10	1	1	0.80	152	30	3	0.5	1	0.46

Table 14: MBCs (mg/L) results at various vancomycin (VA) and daptomycin (DA) concentrations during two different periods.

Period	Total nº of isolates	VA (mg/L)					MBC50	MBC90	Geometric mean	DA (mg/L)			MBC50	MBC90	Geometric mean
		0.5	1	2	4	≥16				≤0.5	1	2			
1990-1999	52	3	5	2	1	41	>16.0	>64.0	17.61	26	25	1	0.5	1	0.70
2000-2009	133	52	73	4	1	3	1	1	0.86	109	20	4	0.5	1	0.46
Total nº of isolates	185	55	78	6	2	44	1	>64.0	2.02	135	45	5	0.5	1	0.52

Both VA and DA MICs decreased from the first period (1990-1999) to the second (2000-2009). A significant decrease on the VA MBC was observed from a geometric mean of 17.61 mg/L to 0.86 mg/L. The clonal composition of analyzed strains in each period was responsible for the observed differences. MIC_{50}/MIC_{90} and MBC_{50}/MBC_{90} values for each clone are shown in Table 15.

Table 15: MIC_{50}/MIC_{90} and MBC_{50}/MBC_{90} values for each clone.

Clone		VA (mg/L)		DA (mg/L)	
		MIC_{50}/MIC_{90}	MBC_{50}/MBC_{90}	MIC_{50}/MIC_{90}	MBC_{50}/MBC_{90}
Iberian	ST247	1 / 2	>16 / >64	≤0.5 / 1	0.5 / 1
Range		0.5 - 2	0.5 - >64	≤0.25 - 1	≤0.25 - 2
CC5	ST5, ST125, ST146	0.5 / 1	1 / 1	0.5 / 0.5	0.5 / 0.5
Range		0.5 - 1	0.25 - 4	0.12 - 2	0.12 - 2
Southern Germany	ST228	1 / 1	1 / 1	0.5 / 0.5	0.5 / 1
EMRSA-15	ST22	0.5 / 1	0.5 / 1	0.5 / 1	0.5 / 2
-	ST8	0.5 / 1	0.5 / 1	0.25 / 0.5	0.25 / 0.5

VA-tolerance was found only in isolates of Iberian clone (43/54; 80%) which account for 23% of the total isolates studied (43/185) (see Table 16). The rate of VA-tolerance is higher among isolates with an MIC of 2mg/L (10/10). MICs and MBCs to VA and DA from the other clones were similar.

Table 16: Vancomycin (VA)-tolerance and association with different MICs.

MIC (mg/L)	Isolates (n)		Tolerance (%)
	VA-tolerance	No VA-tolerance	
≤0.5	0	69	0
1	33	73	31
2	10	0	100
Total	**43**	**142**	**23**

Study 3: Accessory gene regulator function, *rpoB* mutations and vancomycin susceptibility in MRSA blood isolates.

S. aureus accessory gene regulator (*agr*) operon controls the expression of several virulence factors and metabolic pathways. Mutations causing *agr* dysfunction can modify the expression of autolysins and hemolysins and have been associated with reduced vancomycin (VA) bactericidal activity, this situation being described in *agr* type II. In addition, mutations in the *rpoB* gene have been described as contributors to VA resistance in *S. aureu*s. The aim of this work was to study the *agr* function, *rpoB* mutations and VA activity in a selection of MRSA isolates.

The studies were performed in the same MRSA collection used in the previous **study 2:** One-hundred and eighty-five MRSA blood isolates from single pts admitted to HUB from 1990 to 2009, and belonging to the most common MRSA clones endemic in the HUB. Table 17 shows molecular characteristics and number of studied isolates. Negative delta-hemolysin, indicative of dysfunctional *agr* operon, was found in 42% (43/103) of *agr* II isolates and 35% (29/82) of *agr* I. In the *agr* II-group, 67% (29/43) had a VA-MIC of 1 mg/L and in the *agr* I-group, 10% (3/29) had VA-MIC of 2 mg/L.

The *agr* dysfunction was associated with both VA-tolerant and non-VA-tolerant isolates and could be detected in both *agr* types.

Table 17: Vancomycin (VA) activity and *agr* functionality.

agr	MLST-SCC*mec* (n)	VA-MIC (mg/L)	VA-tolerance (n)	Delta-hemolysin	
				Positive (n)	Negative (n)
I	ST247-I and ST1819-I (54)	1.0 – 2.0	43	36	18
I	ST8-IV (15)	0.25 – 1.0	0	11	4
I	ST22-IV (13)	0.5 – 1.0	0	6	7
II	ST228-I (27)	0.5 – 1.0	0	4	23
II	ST5-IV, ST125-IV and ST146-IV (76)	0.5 – 1.0	0	56	20

Mutations in *rpoB*, and their association with VA activity were studied in 47 rifampicin (RA) resistant isolates belonging to ST247-SCC*mec*I-*agr*I (n=20) and ST228-SCC*mec*I-*agr*II (n=27) clones (see Table 18). RA resistance was mainly due to *rpoB* mutation H481N in strains of ST228 (1-4 mg/L) and to H481N plus S529L in strains of ST247 (≥ 8 mg/L). VA MICs of corresponding isolates are shown in the Table 18.

Thus, *rpoB* mutations were detected in both VA-tolerant and non-VA-tolerant isolates.

Table 18: Vancomycin (VA) activity and *rpoB* mutations.

Genotype: ST/SCC*mec*	RA MICs (mg/L)	*rpoB* mutations	VA MICs (mg/L)	No. VA-Tolerant isolates
ST247/I (n=20) *agr* I	≥8 (n=20)	481His→Asn 529Ser→Leu	1 (n=10)	0
			2 (n=10)	10
ST228/I (n=27) *agr* II	≤0.5 (n=2)	none	0.5-1.0 (n=27)	0
	1-4 (n=24)	481His→Asn		
	≥8 (n=1)	481His→Asn 477Ala→Thr		

Study 4: Prevalence of *cfr*-mediated linezolid resistance among MRSA in the HUB.

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Low Prevalence of *Cfr*-Mediated Linezolid Resistance among Methicillin-Resistant *Staphylococcus aureus* in a Spanish Hospital: Case Report on Linezolid Resistance Acquired during Linezolid Therapy

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Abstract

Linezolid is an effective antimicrobial agent to treat methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance to linezolid due to the *cfr* gene is described worldwide. The present study aimed to analyze the prevalence of the *cfr*–mediated linezolid resistance among MRSA clinical isolates in our area. A very low prevalence of *cfr* mediated linezolid resistance was found: only one bacteremic isolate out of 2 215 screened isolates. The only linezolid resistant isolate arose in a patient, previously colonized by MRSA, following linezolid therapy. Despite the low rate of resistance in our area, ongoing surveillance is advisable to avoid the spread of linezolid resistance.

Citation: Sierra JM, Camoez M, Tubau F, Gasch O, Pujol M, et al. (2013) Low Prevalence of *Cfr*-Mediated Linezolid Resistance among Methicillin-Resistant *Staphylococcus aureus* in a Spanish Hospital: Case Report on Linezolid Resistance Acquired during Linezolid Therapy. PLoS ONE 8(3): e59215. doi:10.1371/journal.pone.0059215

Editor: Holger Rohde, Universitätsklinikum Hamburg-Eppendorf, Germany

Received November 13, 2012; **Accepted** February 12, 2013; **Published** March 15, 2013

Funding: This work was supported by a grant from Fondo de Investigación Sanitaria (grant number 10/01783) and by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD06/0008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Linezolid (LZD) is an effective antimicrobial agent to treat MRSA. Resistance to LZD due to the *cfr* gene is described worldwide. Of note, LZD was introduced in the clinical practice in HUB during 2003. The present study (**article 1, Annex I**) aimed to analyse the prevalence of the *cfr*–mediated LZD resistance among MRSA clinical isolates in our area. The *cfr* gene is mostly plasmid-located and confers cross-resistance to phenicols, lincosamides, oxazolidinones, pleromutilines and streptogramin A. Only 16 MRSA isolates (0.7%) had the clindamycin and chloramphenicol resistance profile. LZD MICs were ≤2 mg/L in all but one strain, which showed MIC of 8 mg/L and carried the *cfr* gene (see Table 19). The LZD resistant isolate was also resistant to erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampicin and chloramphenicol and belonged to clone ST228-MRSA-SCC*mec*I with *agr* type II. LZD susceptible MRSA strains also belonged to clone ST228-MRSA-SCC*mec*I. No further spread of the LZD resistant strain to other patients was detected. In our series, overall LZD resistance mediated by the *cfr* gene is very low in this period (0.05%; 1/2,215). Among clindamycin and chloramphenicol resistant strains, *cfr*-mediated LZD resistance was 6.25%. The only LZD resistant isolate arose in a patient, previously colonized by MRSA, following LZD therapy.

Table 19: Linezolid susceptibility by MIC and disc-diffusion in 16 clindamycin and chloramphenicol resistant MRSA isolates and presence of the *cfr* gene (from **Table 1**, **article 1, Annex I**).

Number of strains	Linezolid MIC (mg/L)	Linezolid inhibition zone (mm)	*cfr* gene
15	1-2	29-32	-
1	8	25	+
Control strains (n=2)	8-16	26	+

1.2. Molecular epidemiology description of emergent and endemic MRSA clones using genotypic and proteomic based methods.

Study 5: Molecular characterization of the emergent and endemic MRSA clones by standard typing methods.

Seven hundred and sixteen bloodstream infections were documented from single patients admitted at HUB between 1990 and 2014. Four hundred and eighty-five bacteremic isolates were available for this study. The yearly distribution of these isolates, along with antibiotic resistance percentages are shown in Table 20.

Table 20: Yearly distribution of MRSA blood isolates and trends in antimicrobial resistance of MRSA bacteremic isolates.

	1st Period (1990-1994)	2nd Period (1995-1999)	3rd Period (2000-2004)	4th Period (2005-2009)	5th Period (2010-2014)
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
No. of Blood isolates / Total No	67/134 (50)	34/85 (40)	96/168 (57)	130/163 (80)	158/166 (95)
Resistance to antibiotics					
Ciprofloxacin	64 (96)	33 (97)	91 (95)	126 (97)	148 (93)
Tobramycin	64 (96)	34 (100)	80 (83)	96 (74)	109 (68)
Erythromycin	64 (96)	32 (94)	63 (66)	95 (73)	80 (50)
Clindamycin	64 (96)	32 (94)	41 (43)	66 (51)	29 (18)
Gentamicin	64 (96)	29 (85)	26 (27)	51 (39)	21 (13)
Tetracycline	64 (96)	29 (85)	14 (15)	1 (1)	5 (3)
Rifampicin	67 (85)	28 (82)	20 (21)	43 (33)	3 (2)
Trimethoprim-sulfamethoxazole	1 (1)	2 (6)	0 (0)	0 (0)	0 (0)

To describe the genotypic diversity of this MRSA population, PFGE was performed in all 485 bacteremic isolates. Out of this collection a selection of 328 (328/485; 68%) isolates, including all genetic variants detected by PFGE, was

typed by SCC*mec*, MLST, and *agr.* Additionally, a collection of 257 (257/485; 53%) isolates was typed by *spa* and characterized by PVL and ACME detection. Genetic background of the remaining isolates was inferred by PFGE genotype.

Pulsed-field gel electrophoresis (PFGE) characterization

PFGE analysis showed that 5 PFGE types accounted for 95% (460/485) of the studied strains: PFGE-D;E (198/485; 41%), PFGE-A;B (105/485; 22%), PFGE-C (73/485; 15%), PFGE-F (55/485; 11%) and PFGE-G (29/485; 6%). The remaining 25 (5%) isolates belonged to sporadic profiles. The yearly distribution of the main clonal types is shown in Figure 17.

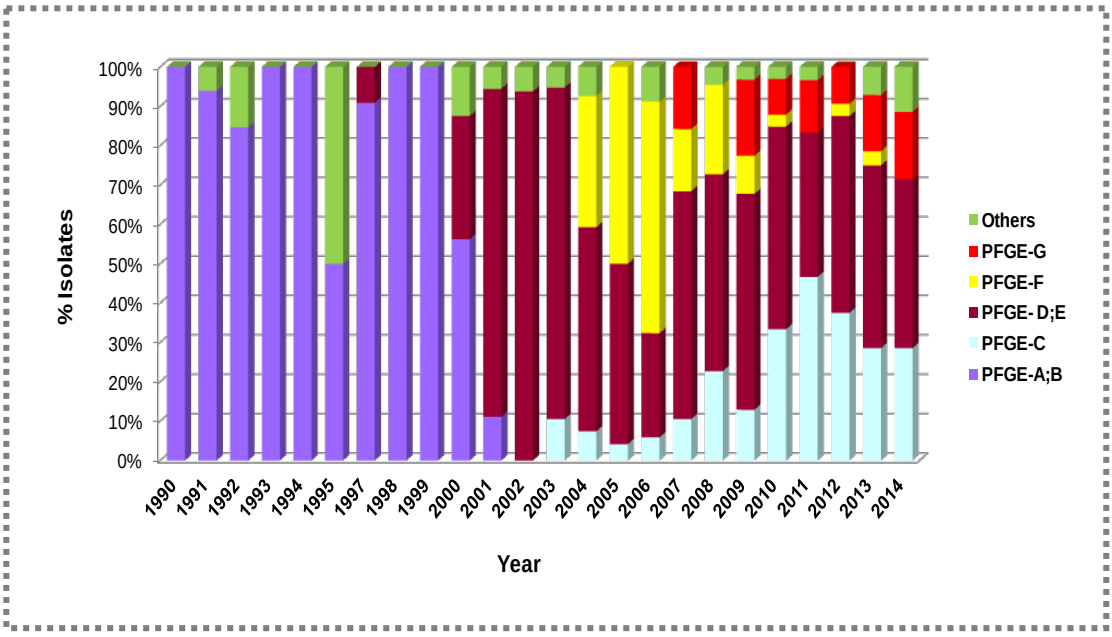

Figure 17: Pulsed-field gel electrophoresis (PFGE) genotypes from 1990 to 2014 in HUB (n=485 bacteremic MRSA isolates) * Isolates of PFGE D and E; A and B had similar PFGE patterns and belonged to same Clonal Complexes by MLST, thus the figures are plotted together.

Staphylococcal cassette chromosome *mec* (SCC*mec*) types

SCC*mec* element was investigated in a selection of 328 MRSA blood isolates and four different polymorphisms were found: type I, II, IV, and V The most common SCC*mec* type was IV (214/328; 65%) followed by SCC*mec* type I (109/328; 33%). Minor SCC*mec* polymorphisms were SCC*mec* type V (4/328; 1.2%) and type II (1/328; 0.3%).

SCC*mec* type IV was included in three major PFGE types (PFGE-D;E, PFGE-C and PFGE-G) accounting 94% (202/214) of the studied isolates, and in 10 minor clones mostly represented by single isolates.

SCC*mec* type I (95%; 104/109) was mainly present in genotypes PFGE-A;B and PFGE-F and in four sporadic clones (accounting for five isolates).

SCC*mec* type V was detected in four isolates (PFGE NT_{SmaI}-MRSA, n=2; PFGE-D;E, n=1 and PFGE-H, n=1) and SCC*mec*II was only present in a minor clone (PFGE-I, n=1).

SCC*mec* type assignments were confirmed by *ccrB* typing in a selection of 62 isolates including representatives of different PFGE and *spa* types All the isolates that present the *ccrB* allotype 412 that was described in a methicillin susceptible coagulase negative isolate (DFT676NR1) belong to clone ST8-SCC*mec*IV-agrI (see Table 21). Two additional isolates, highly related to USA300 (PFGE type C, ST8-SCC*mec*IV-*agr*I) showed the *ccrB* allotype 400. The presumptive SCC*mec* types defined by *ccrB* typing were concordant with the results previously obtained by SCC*mec* multiplex strategy.

Table 21: SCC*mec* analysis by *ccrB* typing.

ccrB allotype	Database reference isolate	Expected database SCC*mec*	Isolates (n)
100	COL	I	18
400	Q2314	IV	35
412	DFT676NR1	MS	8
new allele: allele 100 related	New closest: COL (99.8%)	I	1
Total			**62**

Multilocus sequence typing (MLST)

Molecular typing by MLST of 328 isolates showed that ST247/ST1819-SCC*mec*I (named Iberian clone) was the main clone until 1999 (see Figure 18). This pandemic clone (PFGE-A;B) belonged to CC8 and was found in 58 (58/328; 18%) isolates. Between 1996 and 2003, isolates of CC5 rapidly increased and become prevalent until the end of the studied period (2014). Thus,135 (135/328; 41%) isolates of PFGE-D;E and two isolates of two minor PFGE types (PFGE-L and PFGE-O) clustered in CC5 group and included variants of the paediatric clone: eight isolates of ST5-SCC*mec*IV, 124 of ST125, ST146-SCC*mec*IV, and three single isolates of ST965-SCC*mec*IV, ST1398-SCC*mec*IV and ST2853-SCC*mec*IV.

After 2004, other clones were introduced in HUB such as PFGE-G belonging to ST22-SCC*mec*IV (n=24) and PFGEs-F, -M, -N and -Q, belonging to ST228-SCC*mec*I (n=51).

Since 2010, CC8 (ST8, ST450, ST2840-SCC*mec*IV) became the second major clone circulating in our hospital, accounting for 14% (46/328) of the studied isolates. In this group the following PFGE patterns were identified: PFGEs-C, -P, -USA300 and -USA300/ACME negative.

Recently, sporadic clones (14/328; 4%) emerged in our setting such as ST72-SCC*mec*IV (n=4), ST45-SCC*mec*IV (n=3), ST2222-SCC*mec*IV (n=1), ST36-SCC*mec*II (n=1), ST50-SCC*mec*IV (n=1), ST59-SCC*mec*V (n=1), ST398-SCC*mec*V (n=1), ST3207-SCC*mec*V (n=1) and ST1-SCC*mec*IV (n=1).

The distribution of the major MLST sequence types over time is shown in Figure18.

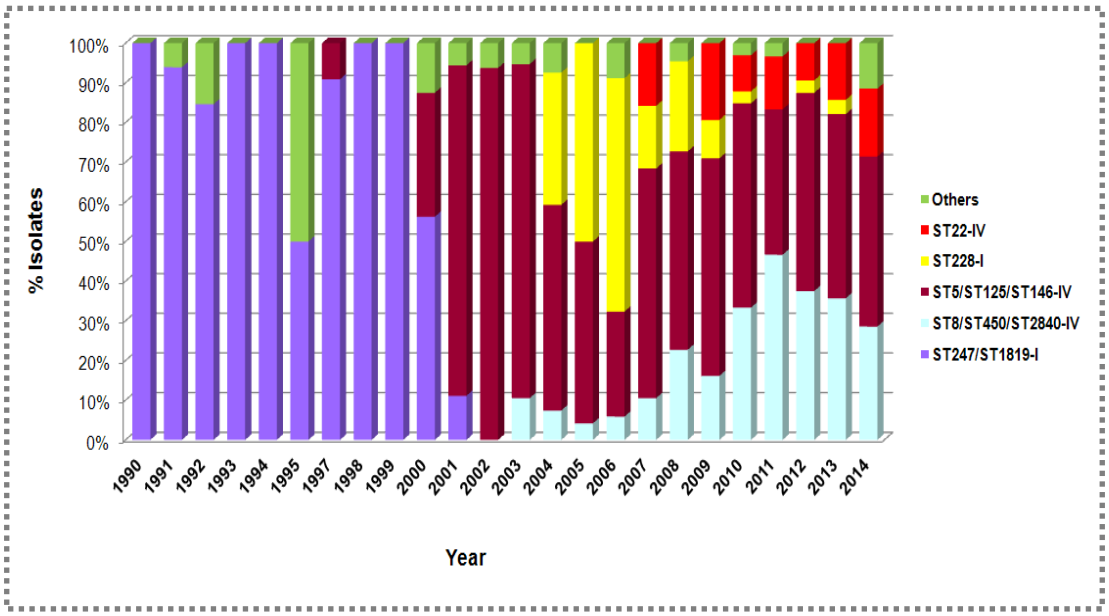

Figure 18: Clonal distribution of the studied strains isolated from 1990 to 2014.

Accesory gene regulator (*agr*) types

The *agr* group was investigated in a selection of 328 MRSA blood isolates and its distribution followed the clonal assignment. Similar proportion of isolates were found in both *agr* polymorphisms type II and I. *agr* type II was present in 187 (57%) of the strains belonging to the major clones PFGE D;E (ST5, ST125, ST146-SCC*mec*IV), PFGE-F (ST228-SCC*mec*I) and to the sporadic clone ST2222-SCC*mec*IV. *agr* type I was detected in 138 (42%) isolates and grouped strains belonging to three major clones PFGE-A;B (ST217/ST1819-SCC*mec*I), PFGE-C (ST8/ST450/ST2840-SCC*mec*IV) and PFGE-G (ST22-SCC*mec*IV)

and three minor clones (ST45-SCC*mec*IV, ST59-SCC*mec*V and ST72-SCC*mec*IV). Only 0.6% of the MRSA strains harboured *agr* type III; these strains belonged to the ST36-SCC*mec*II. One isolate presented *agr* type IV and was associated with ST50-SCC*mec*IV.

Panton-Valentine leukocidine (PVL) genes and arginine catabolic mobile element (ACME).

Among 257 MRSA blood isolates, five were PVL positive, showed PFGE genotype C and belonged to the ST8-SCC*mec*IV-*agr*I (n=4) and ST2840-SCC*mec*IV-*agr*I (n=1). Additionally, two of them presented the typically community acquired genotype of the USA300, one with a positive ACME, the other with negative ACME.

Molecular features and antibiotic resistance patterns (RP) relationship.

Molecular and resistance traits of MRSA endemic clones in HUB over 25 years are summarized in Table 22. In general, PFGE-A;B (ST247/ST1819) was mostly associated (90.5%; 95/105) with the single multiresistant profile RP1. More than half of the strains belonging to PFGE-C (ST8/ST450/ST2840) were associated (60.5%; 46/76) with RP2, RP4 and RP33. PFGE-D;E (ST5/ST125/ST146) was mainly associated (73.2%; 145/198) with four different RPs (RP2, RP4, RP30 and RP33). In contrast, PFGE-F (ST228) showed particularly (96.4%; 53/55) a single multiresistant profile – RP48. Finally, PFGE-G (ST22) was mostly associated with non- multiresistant profiles RP10 and RP30.

Table 22: Molecular features and resistance patterns of 485 MRSA blood strains isolated in HUB during the 1990-2014 period.

RP	Strains n	ST247/ST1819 SCC*mec*I;*agr*I	ST5/ST125/ST146 SCC*mec*IV;*agr*II	ST8/ST450/ST2840 SCC*mec*IV;*agr*I	ST228 SCC*mec*I;*agr*II	ST22 SCC*mec*IV;*agr*I	Others
		PFGE-A;B	PFGE-D;E	PFGE-C	PFGE-F	PFGE-G	
1	95	95	0	0	0	0	0
2	35	0	19	14	0	0	2
3	1	0	0	0	0	0	1
4	69	0	52	17	0	0	0
5	8	7	0	0	0	0	1
7	3	3	0	0	0	0	0
9	9	0	2	7	0	0	0
10	21	0	6	3	0	12	0
12	9	0	1	5	0	0	3
13	3	0	3	0	0	0	0
15	1	0	1	0	0	0	0
17	1	0	0	0	0	0	1
18	2	0	2	0	0	0	0
20	6	0	3	0	0	0	3
21	4	0	4	0	0	0	0
22	1	0	0	0	0	0	1
23	3	0	1	0	1	1	0
24	1	0	1	0	0	0	0
26	13	0	8	5	0	0	0
27	4	0	1	0	0	0	3
30	50	0	28	5	0	14	3
33	61	0	46	15	0	0	0
35	10	0	8	2	0	0	0
37	2	0	0	1	0	1	0
38	8	0	8	0	0	0	0
42	3	0	0	1	0	0	2
48	53	0	0	0	53	0	0
49	1	0	0	0	0	0	1
53	1	0	1	0	0	0	0
56	1	0	0	1	0	0	0
61	1	0	0	0	1	0	0
62	1	0	1	0	0	0	0
65	1	0	1	0	0	0	0
71	1	0	0	0	0	1	0
74	1	0	0	0	0	0	1
90	1	0	1	0	0	0	0
Total	**485**	**105**	**198**	**76**	**55**	**29**	**22**

RP1 (E, CC, G, NN, CIP, RA, TE);
RP2 (E, CC, NN, CIP);
RP3 (E, CC, NN);
RP4 (NN, CIP);
RP5 (E, CC, G, NN, TE, CIP);
RP7 (E, CC, G, NN, TE, SXT, CIP, RA);
RP9 (G, NN, CIP);
RP10 (E, CC, CIP);
RP12 (beta-lactams);
RP13 (E, CC, C, NN, CIP);
RP15 (C, NN, CIP);
RP17(E, CC, G, NN);
RP18 (E, C, G, NN, CIP);
RP20 (E, CC, G, NN, CIP);
RP21 (E, C, CIP);
RP22 (G, NN);
RP23 (E, CC, C, G, NN, CIP);
RP24 (C, NN);
RP26 (E, CIP);
RP27 (NN);
RP30 (CIP);
RP33 (E, NN, CIP);
RP35 (E, G, NN, CIP);
RP37 (E, CC);
RP38 (E, C, NN, CIP);
RP42 (TE);
RP48 (E, CC, G, NN, CIP, RA);
RP49 (E, NN, TE, CIP);
RP53 (E);
RP56 (NN, TE, CIP);
RP61 (G, NN, RA, CIP);
RP62 (E, G, NN, TE, CIP)
RP65 (E, NN, CIP, RA);
RP71 (TE, CIP);
RP74 (E, CC, NN, TE, CIP);
RP90 (NN, TE)

E=Erythromycin; **CC**=Clindamycin;
NN=Tobramycin; **CIP**=Ciprofloxacin;
G=Gentamicin; **C**=Chloranphenicol;
TE=Tetracycline; **RA**=Rifampicin;
SXT=Trimethoprim-sulphamethoxazole

***S. aureus* protein A (*spa*) typing**

All the *spa* sequences were analyzed with the Ridom software and 44 different *spa* types were assigned to the 257 isolates. The overwhelming majority of the isolates (213/257; 83%) belonged to seven different *spa* types: t067 (n=61), t008 (n=40), t051 (n=39), t002 (n=33), t032 (n=16), t041 (n=18) and t200 (n=6). Over time, *spa* type t051 was the most prevalent spa between 1990 and 1999 (34/46; 74%). Since 2000, several *spa* types were detected with similar isolates proportion between them (see Figure 19).

Excellent correlation between *spa* type and MLST type was observed: i) Isolates of CC5 (ST5, ST125, ST146-SCC*mec*IV-*agr*II) presented either *spa* t067 (61/121; 50%) or *spa* t002 (33/121; 27%); ii) isolates of CC8 (ST8, ST450, ST2840-SCC*mec*IV-*agr*I) belonged to *spa* type t008 (36/38; 95%); iii) isolates of ST247 or ST189-SCC*mec*I-*agr*I belonged to *spa* type t051 (39/49; 80%); iv) isolates of ST228-SCC*mec*I-*agr*II belonged to t041 (18/20; 90%); and v) isolates of ST22-SCC*mec*IV-*agr*I belonged to *spa* type t032 (16/21; 76%).

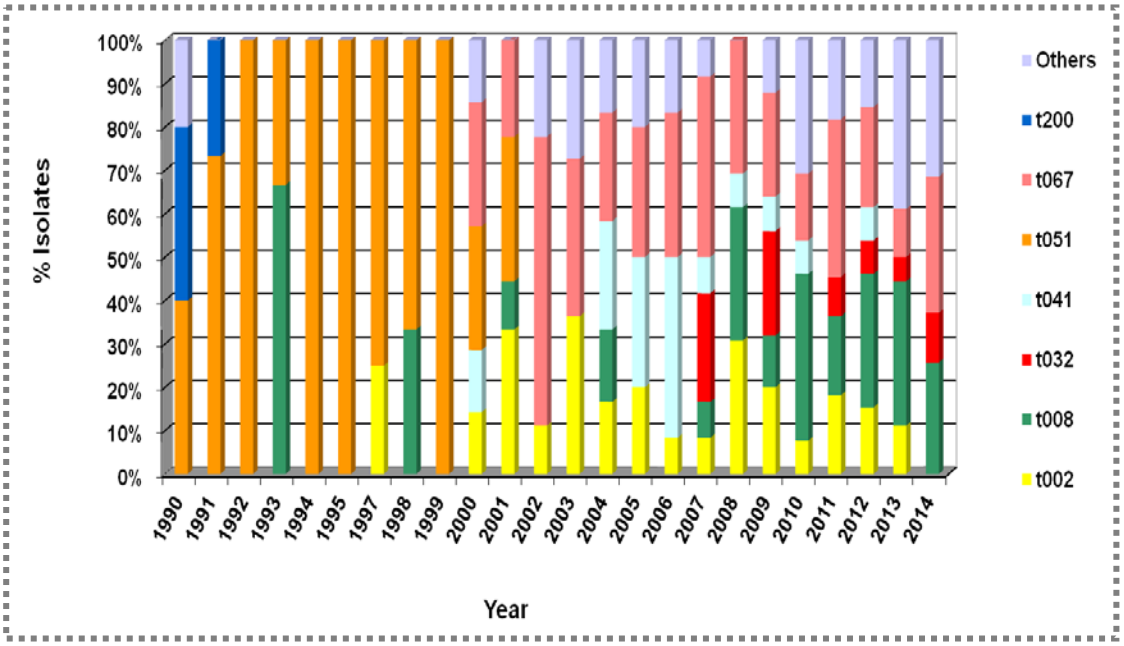

Figure 19: Distribution of *spa* types between 1990-2014 in the HUB.

The BURP algorithm generated by RIDOM software assigned three *spa* clonal complexes (*spa*-CC002, *spa*-CC051/008 and *spa*-CC032/025), six singletons and two *spa* types were excluded from the collection (see Figure 20). The *spa*-CC002 is the prevalent clonal complex since 2002, while the *spa* type t051, the founder of *spa*-CC051/008 was not detected in this period.

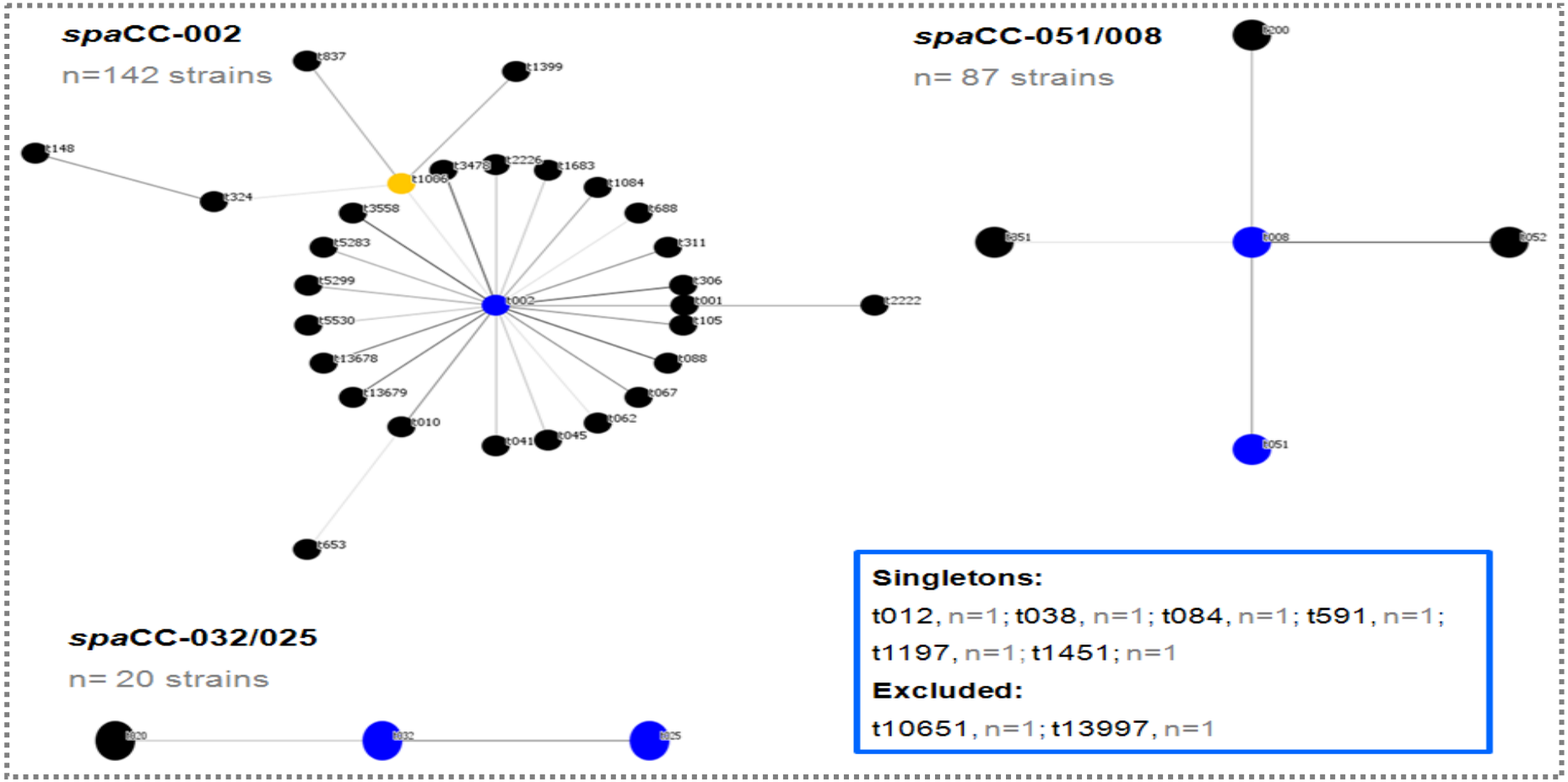

Figure 20: BURP analysis of *spa* data obtained from isolates collected in HUB (1990-2014). *spa* types are represented by dots and the related *spa* types are linked by a line. The shade of the line varies according to the evolutionary cost between the *spa* types, where a darker shade represents a lower evolutionary cost. The ancestor of *spa*-CC cluster is represented in blue dots while yellow dots show a co-founder.

Finally, clonal composition of MRSA isolates that are circulating in the HUB over 25 years (1990-2014) are summarized in Table 23.

Table 23: Clonal composition of MRSA strains isolated in HUB from 1990 to 2014.

CC (%)	ST	SCC*mec*	*agr*	*spa*	PVL (nº of isolates)	ACME (nº of isolates)
5 (53.20)	5	IV	II	t311, t002, t5530	-	-
	125	IV	II	t002, t010, t041, t045, t051, t067, t311, t688, t837, t1006, t1084, t1399, t2226, t5283, t5299	-	-
	146	IV	II	t002, t010, t041, t045, t008, t067, t088, t306, t653, t688, t1084, t2226, t3478, t5283, t13678	-	-
	228	I	II	t001, t041, t067, t2222	-	-
	965	IV	II	t062	-	-
	1398	IV	II	t1683	-	-
	2853	IV	II	t13679	-	-
8 (38.56)	8	IV	I	t008, t351	+ (4)	+ (1)
	72	IV	I	t148, t324	-	-
	247	I	I	t008, t051, t052, t200	-	-
	450	IV	I	t008	-	-
	1819	I	I	t051, t052, t200	-	-
	2840	IV	I	t008	+ (1)	-
22 (5.98)	22	IV	I	t020, t025, t032, t2222	-	-
45 (0.62)	45	IV	I	t038	-	-
398 (0.41)	398	V	I	t1197	-	-
	3207	V	I	t1451	-	-
50 (0.41)	50	IV	IV	Nd	-	-
1 (0.21)	1	IV	III	t591	-	
15 (0.21)	2222	IV	II	t084	-	-
30 (0.21)	36	II	III	t012	-	-
59 (0.21)	59	V	I	Nd	-	-

CC, clonal complex; ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*; *agr*, accessory gene regulator; *spa*, staphylococcal protein A; PVL, leukocidin Panton-Valentine; ACME, arginine catabolic mobile element.

* Nd, not-determined

Numbers in parentheses indicate the number of strains sharing the same characteristic.

Study 6: Prevalence and molecular characterization of livestock-associated MRSA-ST398 isolated in the HUB.

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PLOS ONE

Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* ST398 Resistant to Tetracycline at a Spanish Hospital over 12 Years

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398, associated with livestock animals, was described in 2003 as a new lineage infecting or colonizing humans. We evaluated the prevalence and molecular characteristics of MRSA ST398 isolated in the Hospital Universitari de Bellvitge from January 2000 to June 2011. Tetracycline resistant (Tet-R) MRSA isolates from single patients (pts) were screened by *Sma*I-pulsed field gel electrophoresis (PFGE). Nontypable MRSA strains by *Sma*I (NT_{SmaI})-MRSA were further analysed by *Apa*I-PFGE, *spa*, SCC*mec*, *agr*, MLST typing, and by DNA microarray hybridization. Among 164 pts harboring Tet-R MRSA, NT_{SmaI}-MRSA ST398-*agr*I was found in 33 pts (20%). Although the first pt was detected in 2003, 22/33 pts (67%) were registered in the 2010–2011 period. Ten pts (30%) were infected and cancer was the most frequent underlying disease. In one case, death was due to MRSA-ST398-related infection. Five pulsotypes (A–E) were detected using *Apa*I-PFGE, with type A accounting for 76% of the strains. The majority of the studied isolates presented *spa* type t011 (70%) and SCC*mec* type V (88%). One strain was *spa* negative both by PCR and microarray analysis. Forty-nine percent of the studied isolates showed resistance to 3 or more antibiotic classes, in addition to beta-lactams. Ciprofloxacin resistance was 67%. Tet-R was mediated by *tet*(M) and *tet*(K) in 26 isolates. All isolates lacked Panton-Valentine Leukocidin production, as well as other significant toxins. This study displays the molecular features of MRSA-ST398 clone and shows the increase in tetracycline resistance together with arise in MRSA-ST398 isolates infecting or colonizing patients in our clinical setting.

Citation: Camoez M, Sierra JM, Pujol M, Hornero A, Martin R, et al. (2013) Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* ST398 Resistant to Tetracycline at a Spanish Hospital over 12 Years. PLoS ONE 8(9): e72828. doi:10.1371/journal.pone.0072828

Editor: Holger Rohde, Universitätsklinikum Hamburg-Eppendorf, Germany

Received February 12, 2013; **Accepted** July 14, 2013; **Published** September 5, 2013

Funding: This work was supported by a grant from Fondo de Investigación Sanitaria (grant number 10/01783) and by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD06/0008).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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MRSA-ST398, associated with livestock animals, was described in 2003 as new lineage infecting/colonizing humans. Resistance to tetracycline (Tet-R), commonly used in pig farming, and the inability to be typed by *SmaI* restriction (NT_{SmaI}) are characteristic traits of MRSA-ST398 isolates. The aim of this study (**article 2, Annex I**) was to evaluate the prevalence and molecular characteristics of MRSA-ST398 isolated in the HUB (2000-2011).

Among 164 Tet-R MRSA isolated between 2000 and 2011 from single patients, 33 (20%) NT_{SmaI}-MRSA strains were found. All 33 isolates belonged to ST398 and showed an *agr* type I. The first MRSA-ST398 isolate was identified in 2003. Yearly distribution of MRSA-ST398 isolates is shown in Figure 21. It is noted that out of 33 MRSA-ST398, 22 (67%) were isolated during the 2010-2011 period.

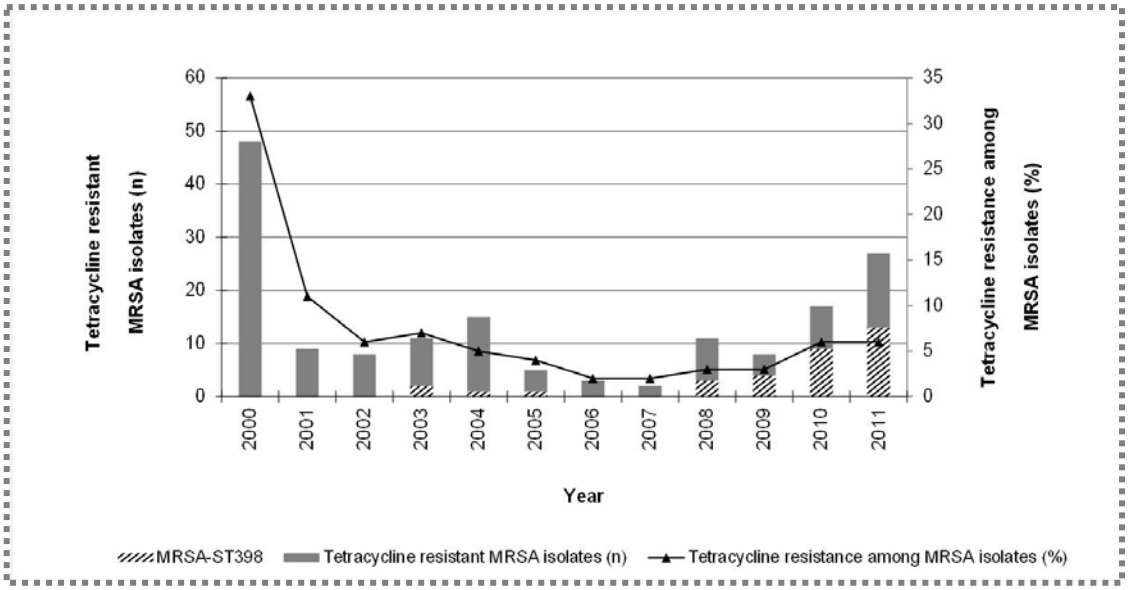
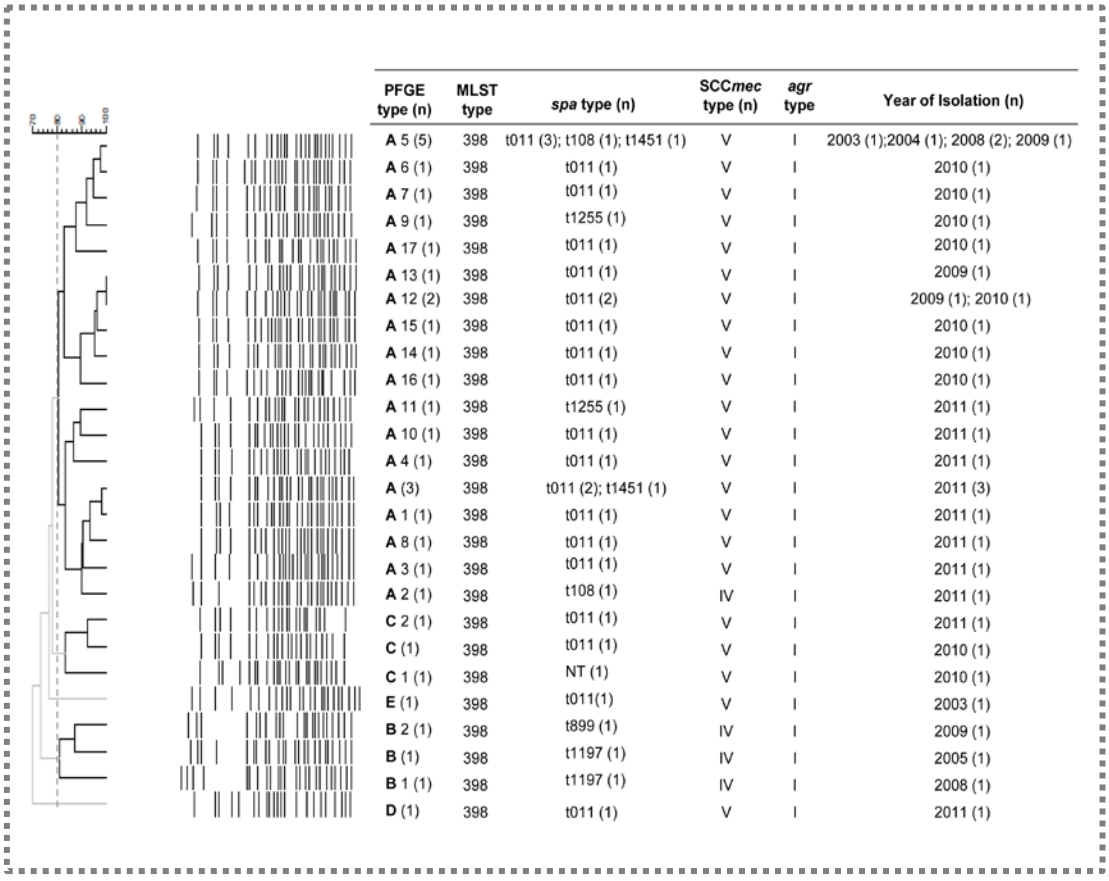


Figure 21: Distribution of 164 tetracycline resistant MRSA isolates from 2000 to 2011 in HUB (from **Figure 1, article 2, Annex I**).

Digestions with *ApaI* restriction of MRSA-ST398 isolates provided five unrelated pulsotypes (A – E) using a cut-off at 80% similarity (see Figure 22).

Among these five major clusters, type A accounted for 76% (25/33) of the studied strains. SCC*mec* type V was carried by 29 (88%) isolates and SCC*mec* type IV was only found in four isolates. A total of six different *spa* types were detected, with t011 as the dominant type present in 23 (70%) isolates. The other *spa* types identified were: t1255 (n=2), t1197 (n=2), t108 (n=2), t1451 (n=2) and t899 (n=1). One strain was *spa* negative both by PCR and microarray analysis (Figure 22). The BURP algorithm assigned all spa types, except singleton t899, to *Spa*-CC011 (n=31; 94%).



PFGE type (n)	MLST type	*spa* type (n)	SCC*mec* type (n)	*agr* type	Year of Isolation (n)
A 5 (5)	398	t011 (3); t108 (1); t1451 (1)	V	I	2003 (1);2004 (1); 2008 (2); 2009 (1)
A 6 (1)	398	t011 (1)	V	I	2010 (1)
A 7 (1)	398	t011 (1)	V	I	2010 (1)
A 9 (1)	398	t1255 (1)	V	I	2010 (1)
A 17 (1)	398	t011 (1)	V	I	2010 (1)
A 13 (1)	398	t011 (1)	V	I	2009 (1)
A 12 (2)	398	t011 (2)	V	I	2009 (1); 2010 (1)
A 15 (1)	398	t011 (1)	V	I	2010 (1)
A 14 (1)	398	t011 (1)	V	I	2010 (1)
A 16 (1)	398	t011 (1)	V	I	2010 (1)
A 11 (1)	398	t1255 (1)	V	I	2011 (1)
A 10 (1)	398	t011 (1)	V	I	2011 (1)
A 4 (1)	398	t011 (1)	V	I	2011 (1)
A (3)	398	t011 (2); t1451 (1)	V	I	2011 (3)
A 1 (1)	398	t011 (1)	V	I	2011 (1)
A 8 (1)	398	t011 (1)	V	I	2011 (1)
A 3 (1)	398	t011 (1)	V	I	2011 (1)
A 2 (1)	398	t108 (1)	IV	I	2011 (1)
C 2 (1)	398	t011 (1)	V	I	2011 (1)
C (1)	398	t011 (1)	V	I	2010 (1)
C 1 (1)	398	NT (1)	V	I	2010 (1)
E (1)	398	t011(1)	V	I	2003 (1)
B 2 (1)	398	t899 (1)	IV	I	2009 (1)
B (1)	398	t1197 (1)	IV	I	2005 (1)
B 1 (1)	398	t1197 (1)	IV	I	2008 (1)
D (1)	398	t011 (1)	V	I	2011 (1)

Figure 22: Cluster analysis of Pulsed-field Gel Electrophoresis (PFGE) *Apa*I macrorestriction fragments of methicillin-resistant *Staphylococcus aureus* ST398 isolates followed by multilocus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome (SCC*mec*), accessory gene regulator (*agr*) typing and year of isolation. For dendogram construction, optimization and band position tolerance were both set at 0.7%. The cut-off value to define the PFGE patterns was set at 80% similarity (from **Figure 2, article 2, Annex I**).

The percentage of antibiotic resistance among the 33 isolates of ST398 was as follows: resistance to erythromycin was found in 11 isolates (33%), to clindamycin in 16 (48%), to tobramycin in 8 (24%) and to ciprofloxacin in 22 (67%). No resistance was found to vancomycin, daptomycin, rifampicin or mupirocin. Fourteen antibiotic resistance patterns were found among the MRSA-ST398 isolates (Table 24). The most frequent combination of resistances were: tetracycline plus ciprofloxacin (8/33; 24%) and tetracycline, ciprofloxacin, erythromycin plus clindamycin (6/33; 18%), with 49% (16/33) of the isolates being resistant to three or more antibiotic groups, in addition to beta-lactams.

Antibiotic resistance genes, as determined by DNA hybridization, are shown in Table 24.

Table 24: Antibiotic resistance patterns and resistance genes of the 33 MRSA-ST398 isolates recovered in Hospital Universitari de Bellvitge, 2000-2011 (from **Table 1, article 2, Annex I**).

Resistance pattern (n)	Tetracycline resistance genes		Other resistance genes					No. Isolates
	tet(K)	*tet(M)*	*erm(A)*	*erm(C)*	*aacA-aphD*	*aadD*	*cat*	
TET, CIP (8)	+	+	-	-	-	-	-	6
	-	+	-	-	-	-	-	2
TET (7)	+	+	-	-	-	-	-	3
	-	+	-	-	-	-	-	3
	+	-	-	-	-	-	-	1
ERY, CLI, TET, CIP (6)	+	+	-	+	-	-	-	6
CLI, TET, CIP (2)	+	+	-	-	-	-	-	2
CLI, TOB, TET, CIP (1)	+	+	-	-	-	+	-	1
ERY, CLI, GEN, TOB, TET, CIP (1)	+	+	+	+	+	-	-	1
ERY, CLI, TOB, TET, CIP(1)	+	+	-	-	-	+	-	1
CLI, GEN, TOB, TET, CIP, SYN (1)	+	+	-	-	+	+	-	1
ERY, CLI, CLO, TOB, TET (1)	+	+	-	+	-	+	+	1
ERY, CLI, TOB, TET (1)	+	+	-	+	-	+	-	1
ERY, CLI, TOB, TET, SXT, CIP (1)	+	+	-	+	-	+	-	1
TOB, TET, CIP (1)	-	+	-	-	-	+	-	1
CLI, TET (1)	+	+	-	-	-	-	-	1
CLO, TET (1)	+	+	-	-	-	-	+	1
Total No of Isolates								33

TET, tetracycline; CIP, ciprofloxacin; CLI, clindamycin; TOB, tobramycin; GEN, gentamicin; SYN, synercid; ERY, erythromycin; CLO, chloramphenicol; SXT, sulfamethoxazole-trimethoprim.

All 33 strains harboured *mec*A and the beta-lactamase gene *bla*Z. Tetracycline resistance in most of the cases (26/33) was mediated by both *tet(*K) and *tet(*M) genes. In addition to tetracycline resistance, erythromycin and clindamycin combined resistance was seen in 10 (30%) isolates carrying the gene *erm*(C)– one of these isolates harbored *erm*(A) in addition to *erm*(C)-, and one isolate was negative for these genes. Other macrolide and lincosamide resistance genes reported to occur in staphylococci such as *erm*(B)*, msr*(A), *mph*(C), *vga*(A) and *lnu*(A) were negative in this subgroup of strains. Five isolates (15%) showed an unusual erythromycin-susceptibility/clindamycin-resistance pattern. No genes associated with this resistance phenotype such as *vga*(A) or *lnu*(A) were identified by microarray in the MRSA-ST398 strains studied. Two isolates showing resistance to gentamicin harbored the gene *aacA/aphD*. Tobramycin resistance was mediated by *aacA/aphD* (1/8) and by *aadD* (7/8) genes. Chloramphenicol resistance was detected in two strains carrying the *cat* gene.

Regarding the presence of genes coding for virulence factors, none of the MRSA-ST398 isolates harbored the genes encoding PVL (*luK-F/lukS*), enterotoxins (*sea* to *ser*), exfoliative toxins (*etA*/*etB*/*etD*), *egc* cluster (*seg/sei/sem/sen/seo/seu*) or TSST (*tst*). A single isolate carried the *seb* gene encoding enterotoxin B. The vast majority of the strains were positive for haemolysins genes *hla*, *hlb, hld*, *hlgA*, *hlgB* and *hlgC*. All but one, were positive for the *cna* gene responsible by the collagen-binding adhesion. Genes carried on mobile genetic elements and involved in immune evasion such as *scn* (staphylococcal complement inhibitor), *sak* (staphylokinase) and *chp* (chemotaxis inhibitory protein) were identified in a single isolate of *spa* type

t899. Fibronectin-binding protein A gene (*fbnA*) was detected in 9/33 (27%) of the isolates. All isolates carried genes codifying capsule type 5.

The clinical characteristics of patients infected or colonized by MRSA-ST398 are shown in Table 25.

Table 25: Clinical features of 33 patients colonized or infected by MRSA-ST398 (from **Table 2, article 2, Annex I**).

	No. (%) or mean ± SD with variable	
Men	27 (81)	
Age, years (range)	65 (41-92) ± 12.9	
Rural area	24 (77)	
Underlying conditions		
Cancer	11 (33)	
Sample Source	**Colonization**	**Infection**
Blood	0 (0)	1 (3)
Respiratory tract	1 (3)	4 (12)
Skin and Wound	4 (12)	4 (12)
Nares	18 (55)	0 (0)
Central Nervous System	0 (0)	1 (3)
Total number (%)	**23** (70)	**10** (30)

In total, there were 27 men (81.8%) and six women (18.2%) with a mean age of 65 years (range 41 to 92). In 23 patients (70%), the isolation of MRSA-ST398 was considered colonization, more frequently as result of active surveillance for nasal MRSA colonization (17/23; 78%). MRSA-ST398 was causing infection in 10 patients. Eight patients experienced non-invasive infections: in four cases the source of the infection was the respiratory tract and in the other four cases the patients experienced non-invasive skin and soft tissue (SST) infections. Two patients, with prior community-acquired MRSA-

ST398 nasal colonization, went through invasive infections: one of them suffered a catheter-related bacteremia and the other a subdural empyema following a surgical drainage of a subdural haemorrhage. The patient with the catheter-related bacteremia was the sole case where death could be related to the MRSA-ST398 infection. The most frequent underlying disease was cancer, with this condition found in 11 patients, of whom six were colonized and five were infected by MRSA-ST398.

The increased rate of MRSA-ST398 clone observed from 2010 to 2011 in our setting, lead us to the prospective surveillance of this microorganism during 2012 and 2013 (**article 3, Annex I**).

Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Barcelona, Spain, 2012-2013.

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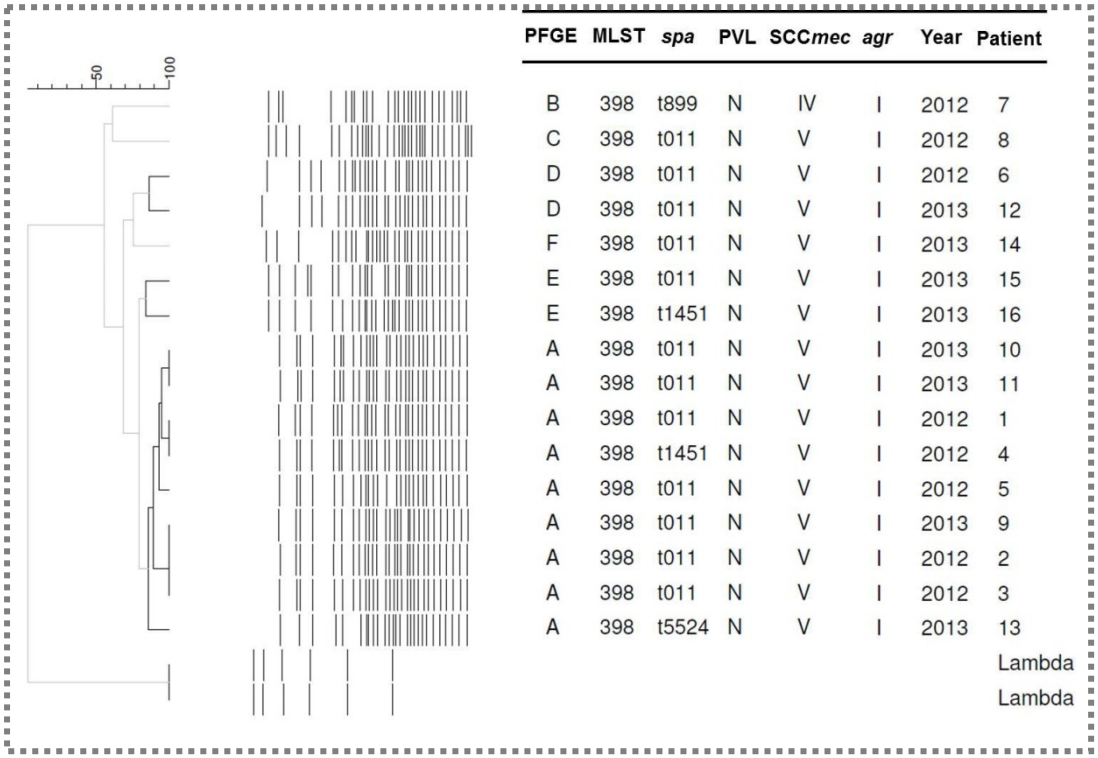
(Submitted to European Journal of Clinical Microbiology & Infectious Diseases)

Of the 1,127 MRSA strains isolated during the 2012-2013 period from single patients, 16 (1.4%) NT*Sma*I-MRSA isolates were found. All 16 isolates share MLST ST398 and *agr* type I.

Six unrelated pulsotypes (A-F) were provided by *Apa*I-PFGE restriction, with type A accounting 56% (9/16) of the strains (see Figure 23). All isolates carried the SCC*mec* type V with the exception of one isolate that carried the SCC*mec* type IV. Further analysis by *spa* typing revealed 4 different types, with t011 as the most frequent type (n=12; 75%), followed by t1451 (n=2; 13%), t5524 (n=1; 6%) and t899 (n=1; 6%). The BURP cluster analysis assigned all *spa* types, except singleton t899, to *spa*-CC011 (n=15; 94%).

Susceptibility testing identified that all MRSA-ST398 isolates were resistant to tetracycline, 81% (13/16) to ciprofloxacin, 38% (6/16) to clindamycin, 31% (5/16) to erythromycin and 25% (4/16) to tobramycin. All isolates were susceptible to glycopeptides, daptomycin, rifampin and mupirocin. Six different antibiotic resistance patterns were found among the MRSA-ST398 isolates (see Table 26), the most common one being the combination of resistance to tetracycline plus ciprofloxacin (8/16; 50%). By DNA microarray Tet-R was (12/16) mediated by both *tet*(M) and *tet*(K) genes in 12 isolates (12/16; 75%) (see Table 26). All the strains carried *mec*A and harbored the beta-lactamase gene *bla*Z. Combined resistance to macrolides-lincosamides was detected in 5 isolates carrying the *erm*(C) (4/5) and *erm*(C) plus *erm*(B) (1/5) genes. A single isolate showed an uncommon erythromycin-susceptible/clindamycin resistance profile with the absence of typical genes associated to this phenotype such as *vga*(A) or *lnu*(A). Gentamicin resistance was detected in two isolates: one carried the *aacA-aphD* gene and the other one carried both *aacA-aphD* and

aadD genes. In four isolates tobramycin resistance was mediated by *aacA-aphD* (1/4), *aacA-aphD* plus *aadD* (1/4), and *aadD* (2/4) genes. The *czr*C, cadmium and zinc resistance genes, were identified in all ST398 isolates carrying the SCC*mec* type V.



PFGE	MLST	*spa*	PVL	SCC*mec*	*agr*	Year	Patient
B	398	t899	N	IV	I	2012	7
C	398	t011	N	V	I	2012	8
D	398	t011	N	V	I	2012	6
D	398	t011	N	V	I	2013	12
F	398	t011	N	V	I	2013	14
E	398	t011	N	V	I	2013	15
E	398	t1451	N	V	I	2013	16
A	398	t011	N	V	I	2013	10
A	398	t011	N	V	I	2013	11
A	398	t011	N	V	I	2012	1
A	398	t1451	N	V	I	2012	4
A	398	t011	N	V	I	2012	5
A	398	t011	N	V	I	2013	9
A	398	t011	N	V	I	2012	2
A	398	t011	N	V	I	2012	3
A	398	t5524	N	V	I	2013	13
							Lambda
							Lambda

Figure 23: Dendogram of *Apa*I-Pulsed-field Gel Electrophoresis (PFGE) representing the genetic relationship among 16 MRSA-ST398 isolates. Multilocus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome (SCC*mec*) and accessory gene regulator (*agr*) typing, year of isolation and patient data were shown at the right side of the figure. Lambda ladder marker was included in two lanes to normalize the PFGE patterns. The similarities among the fingerprints were calculated using the Jaccard coefficient (optimization, 0%; band position tolerance, 0.7%). The cut-off value to define the PFGE patterns was set as 80% similarity (from **Figure S1, article 3, Annex I**).

Concerning the virulence content of MRSA-ST398 isolates, DNA hybridization was negative for genes encoding PVL (*lukS-PV*/*lukF-PV*), enterotoxins (*sea* to *ser*), exfoliative toxins (*etA*/*etB*/*etD*), *egc* cluster (*seg*/*sei*/*sem*/*seo*/*seu*) or TSST (*tst*). All the strains were positive for the haemolysins genes *hla*, *hlb*, *hld*, *hlgA*, *hlgB* and *hlgC* and carried genes codifying capsule 5 *(capH5, capJ5, capK5).*

Table 26: Characteristics of MRSA-ST398 isolated in southern Barcelona metropolitan area, Spain 2012-2013 (n=16) (from **Table 1, article 3, Annex I**)

PFGE	SCC*mec*	*agr*	*spa*	Resistance phenotype[a]	Resistance genotype	Virulence genes[b]	Biofilm genes[c]	MSCRAMM genes[d]	Patient[e]	No. Isolates
A	V	I	t011	TET	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	bbp, clfA, clfB, cna, fnbA	1	1
				TET, CIP	*tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	2;3	2
					tetK, tetM, czrC	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	5;10	2
				ERY, CLI, TOB, TET, CIP	*tetK, tetM, ermB, ermC, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB,*	9	1
				CLI, GEN, TOB, TET, CIP	*tetK, tetM, aacA/aphD, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	11	1
			t1451	ERY, CLI, TET, CIP	*tetK, tetM, ermC, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	4	1
			t5524	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	13	1
B	IV	I	t899	TET, CIP	*tetM*	*hlgA, hlgB, hlgC, hla, hld, scn, sak, chp*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	7	1
C	V	I	t011	TET	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	8	1
D	V	I	t011	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld,*	icaA, icaC, icaD	*clfA, clfB, can, fnbA, fnbB*	6	1
				ERY, CLI, TET, CIP	*tetK, ermC, czrC*	*hlgA, hlgB, hlgC, hla, hld, scn, sak, chp*	icaA, icaC, icaD	*clfA, clfB, cna, fnbA, fnbB*	12	1
E	V	I	t011	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	15	1
			t1451	ERY, CLI, TOB, TET, CIP	*tetK, tetM, ermC, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, clfA, clfB, cna, fnbA, fnbB*	16	1
F	V	I	t011	ERY, CLI, GEN, TOB, TET	*tetK, tetM, ermC, aacA/aphD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*clfA, clfB, cna, fnbA, fnbB*	14	1
Total N° of Isolates										**16**

[a]TET, tetracycline; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin.

[b] *hlgA,* gamma-hemolysin component A; *hglB,* gamma-hemolysin component B; *hglC*, gamma-hemolysin component C; *hla,* alpha-hemolysin*; hld,* delta-hemolysin*; scn,* staphylococcal complement inhibitor*; sak,* staphylokinase*; chp,* chemotaxis inhibitory protein.

[c] *icaA,* intercellular adhesion biofilm gene A; *icaC,* intercellular adhesion biofilm gene C; *icaD,* intercellular adhesion biofilm gene C.

[d] *bbp*, sialoprotein binding protein; *clfA,* clumping factor A; *clfB,* clumping factor B; *cna,* collagen binding protein; *fnbA,* fibronectin binding protein A; *fnbB,* fibronectin binding protein B

[e] Patient code

The biofilm formation and MSCRAMM (microbial surface components recognizing adhesive matrix molecules) carriage genes was similar among all isolates including *icaACD* (intercellular adhesion biofilm genes A, C, and D), *cflA* and *cflB* (clumping factors A and B), *cna* (collagen-binding protein), *fn*bA and f*nbB* (fibronectin-binding proteins A and B). Additionally, our study revealed that all isolates lacked *sasG* (*S. aureus* surface protein G) gene and 13 isolates (13/16; 81%) were positive for the *bbp* (bone sialoprotein-binding protein) gene. Two isolates carried genes encoding proteins that were involved in human-specific innate immune system such as staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein (*chp*) and staphylokinase (*sak*) (see Table 26).

Clinical features of the 16 patients colonized or infected by MRSA-ST398 were reviewed. No significant difference between gender was observed (man, n=9; women, n=7) and the mean age was 58 years (range 33 to 81). Fourteen patients underwent surgical procedures during admission. The most common underlying disease was cancer (7/16; 44%). In 10 patients (10/16; 63%), MRSA-ST398 was associated with colonization. Six patients (6/16; 38%) were infected: four experienced non-invasive SSTIs, one a bacteremia and the other one a surgical bone infection (see Table 27). Prior nasal colonization by MRSA-ST398 was detected in four infected patients (two SSTIs, one bacteremia and one bone infection). Three cases (two SSTIs and one colonization detected by tracheostomy swab) were hospital acquired, since all three had the nasal swab taken at admission negative for MRSA. No epidemiological links could be identified among the three patients with hospital acquired strains. In the remaining 13 cases, acquisition of colonization was ascribed to the community.

Table 27: Patients colonized or infected by MRSA-ST398 in southern Barcelona metropolitan area, Spain 2012-2013 (n=16) (from **Table 2, article 3, Annex I**).

Patient	Gender ; Age (years)	Comorbidities[a]	Sample source[b]	Patient Status[c]	Previous MRSA-ST398 carriage	Persistence (months)[d]	Acquisition[e]
1	F ; 33	Obesity	Nares	C	No	-	CA
2	M ; 54	RA	Nares	C	No	-	CA
3	M ; 45	DM, HIV, HCV, RF	Nares	C	No	-	CA
4	M ; 72	DM, CD	SST	I	Yes	7	CA
5	M ; 58	DM, COPD, Cancer	SST	I	Yes	29	CA
6	F ; 59	HCV, HT	Nares	C	No	66	CA
7	F ; 54	Cancer	SST	I	No	-	HA
8	M ; 65	Obesity, DM, Cancer	Bone	I	Yes	1	CA
9	M ; 59	Cancer	Nares	C	No	20	CA
10	F ; 47	NA	Nares	C	No	-	CA
11	F ; 42	CD	SST	I	No	-	HA
12	F ; 67	DM, HBV, CD, Cancer	Nares	C	No	-	CA
13	M ; 81	CD, Cancer	Nares	C	No	5	CA
14	M ; 78	CD	Tracheotomy	C	No	-	HA
15	F ; 40	Cancer	Nares	C	No	-	CA
16	M ; 64	COPD	Blood	I	Yes	1	CA

[a] *RA, rheumatoid arthritis;* DM, diabetes mellitus; HIV, *human immunodeficiency virus; HCV, hepatitis C virus; RF, renal failure; CD, cardiovascular disease;* COPD, chronic *obstructive pulmonary disease;* HT, *hepatic transplantation; NA, not available; HBV, hepatitis B virus*

[b] STT, skin and soft tissue

[c] C, colonization; I, Infection

[d] Hyphens stand for negative persistence

[e] CA, community-acquired; HA, hospital-acquired

In five patients (two with SSTIs and three showing only nasal colonization), nasal swabs were repeatedly positive for MRSA-ST398 for a mean period of 25 months (range 5-66 months) (see Table 27).Compared to the 2010-2011 period when 48% (22/46) of the Tet-R MRSA belonged to clone MRSA-ST398, the current period of study 2012-2013 showed a similar proportion of Tet-R MRSA belonging to ST398: 37% (16/43). Referred to the total number of MRSA isolates, in the 2010-2011 period MRSA-ST398 represented 2.0 % (22/1119) of MRSA isolates. This proportion in the 2012-2013 period was 1.4% (16/1127).

Study 7: Automated categorization of MRSA clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry.

ORIGINAL ARTICLE BACTERIOLOGY

Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry

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Abstract

Early identification of methicillin-resistant *Staphylococcus aureus* (MRSA) dominant clones involved in infection and initiation of adequate infection control measures are essential to limit MRSA spread and understand MRSA population dynamics. In this study we evaluated the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) for the automated discrimination of the major MRSA lineages (clonal complexes, CC) identified in our hospital during a 20-year period (1990–2009). A collection of 82 well-characterized MRSA isolates belonging to the four main CCs (CC5, CC8, CC22 and CC398) was split into a reference set (n = 36) and a validation set (n = 46) to generate pattern recognition models using the ClinProTools software for the identification of MALDI-TOF/MS biomarker peaks. The supervised neural network (SNN) model showed the best performance compared with two other models, with sensitivity and specificity values of 100% and 99.11%, respectively. Eleven peaks (m/z range: 3278–6592) with the highest separation power were identified and used to differentiate all four CCs. Validation of the SNN model using ClinProTools resulted in a positive predictive value (PPV) of 99.6%. The specific contribution of each peak to the model was used to generate subtyping reference signatures for automated subtyping using the BioTyper software, which successfully classified MRSA isolates into their corresponding CCs with a PPV of 98.9%. In conclusion, we find this novel automated MALDI-TOF/MS approach to be a promising, powerful and reliable tool for *S. aureus* typing.

Keywords: Clonal lineages, epidemiology, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, methicillin-resistant *Staphylococcus aureus*, typing

Original Submission: 6 August 2015; **Revised Submission:** 28 September 2015; **Accepted:** 5 October 2015

Editor: D. Raoult

Article published online: XXX

Early identification of methicillin-resistant *Staphylococcus aureus* (MRSA) dominant clones involved in infection and initiation of adequate infection control measures are essential to limit MRSA spread and understand MRSA population dynamics. In this study (**article 4, Annex I**) we evaluated the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) for the automated discrimination of the major MRSA lineages (clonal complexes, CC) identified in our hospital during a 20-year period (1990-2009).

Eighty-two MRSA clinical isolates representative of the four major MRSA CCs in our hospital were used to identify CC-specific biomarker peaks for MALDI-TOF/MS-based subtyping (see Table 28). Isolates were divided in two sets. i) A reference set of 36 isolates belonging to eight different sequence types (ST): ST146 (n=5), ST125 (n=5), ST228 (n=5) from CC5, ST22 (n=5) from CC22, ST398 (n=5) from CC398, and ST8 (n=5), ST247 (n=4), ST1819 (n=2) from CC8 and, ii) A validation set of 46 isolates belonging to six different ST and classified within the same CC as those of the reference set: ST146 (n=5), ST125 (n=5), ST228 (n=5) of CC5, ST22 (n=4) of CC22, ST398 (n=12) of CC398 and ST8 (n=15) of CC8.

An additional set of 13 isolates (see Table 28) representing sporadic MRSA clones isolated in our hospital were also included: ST1 (n=2) of CC1, ST30 (n=2), ST36 (n=1), ST714 (n=1), ST1870 (n=1) of CC30, ST45 (n=1), ST1871 (n=1) of CC45, ST72 (n=3) of CC72 and ST88 (n=1) of CC80.

Table 28: Molecular features of all isolates included in the study (n=95) (from **Table S1, article 4, Annex I**).

	CC[a] (n)	ST[b]	*agr*[c]	SCC*mec*[d]	PVL[e]	Nº Isolates
Reference set	5	125	II	IV	N	5
		146	II	IV	N	5
		228	II	I	N	5
	8	8	I	IV	N	5
		247	I	I	N	4
		1819	I	I	N	2
	22	22	I	IV	N	5
	398	398	I	IV	N	2
			I	V	N	3
		Total				**36**
Validation set	5	125	II	IV	N	5
		146	II	IV	N	5
		228	II	I	N	5
	8	8	I	IV	P	15
	22	22	I	IV	N	4
	398	398	I	IV	N	1
			I	V	N	11
		Total				**46**
Sporadic isolates	1	1	III	IV	N	2
	30	30	III	II	N	1
			III	IV	N	1
		36	III	II	N	1
		714	III	IV	N	1
		1870	III	II	N	1
	45	45	I	IV	N	1
		1871	I	IV	N	1
	72	72	I	IV	N	3
	80	88	III	II	N	1
		Total				**13**

[a]CC, clonal complex;

[b]ST, sequence type;

[c]*agr*, allele type of accessory gene regulator;

[d]SCC*mec*, staphylococcal cassette chromosome *mec*;

[e]PVL, Panton-Valentine leukocidin.

Generation and validation of pattern recognition models

The acquired raw spectra for each isolate of the reference set were loaded into the ClinProTools software and grouped according to their corresponding CC. The average spectrum from each CC was calculated and used to generate pattern recognition models based on the GA, SNN and QC algorithms to identify an optimal set of peaks for the best class separation of the model generation spectra. The SNN algorithm yielded the model with the highest recognition capability and cross validation values (100% and 99.11%, respectively, Table

29) and was therefore chosen to provide an optimal set of peaks for class discrimination.

Table 29: Performance of the pattern recognition models for the differentiation of the main MRSA clonal lineages generated by ClinProTools using the 36 strains from the reference set (from **Table 1, article 4, Annex I**).

Model	Max. peaks[a] (n)	Selected peaks[b] (n)	RC (%)	CV (%)
SNN	30	11	100	99.11
GA	30	7	100	94.53
QC	30	18	96.43	81.25

SNN, supervised neural network; GA, genetic algorithm; QC, quick classifier; RC, recognition capability; CV, cross-validation

[a] maximum number of peaks for model generation.

[b] number of peaks selected by the model.

The model identified 11 peaks ranging from 3,278 to 6,592 *m/z* values as well as the specific contribution of each peak to the model (see Table 30). Eight out of the 11 peaks showed low *p* values (≤0.05) for the Anderson-Darling test evidencing the non-normal distribution of these data (see Table 30) and, thus, the *p* value of the Wilcoxon/Krustal-Wallis was preferred over the *p* value of the t-/ANOVA test to consider them as informative peaks. The remaining 3 peaks showed *p* values for the Anderson-Darling test >0.05 (normally distributed); hence, the t-/ANOVA was considered instead of Wilcoxon/Krustal-Wallis test (Table 30). The statistical analysis showed that the intensity differences of the individual peaks were statistically significant, suggesting their potentiality to discriminate spectra from all four CCs.

Table 30: ClinProTools peak statistics for the eleven peaks of interest[a] (from **Table 3, article 4, Annex I**)

Peak Number	Mass	Weight	DAve	PTTA	PWKW	PAD	Ave1	Ave2	Ave3	Ave4
12	3278.56	0.01575683258213178	2.99	0.0000272	0.00934	< 0.000001	2.64	2.51	2.7	5.51
16	3445.33	0.01537836986752119	30.57	0.000136	0.00116	0.164	15.93	27.23	46.5	37.35
20	3876.68	0.01770379511151569	4.73	0.0383	0.00137	< 0.000001	1.16	5.89	5.88	2
21	3891.54	0.1795355741539599	4.37	0.0000535	0.000839	0.000109	4.95	0.89	1.08	0.58
32	4514.26	0.1323120474380986	3.16	0.0000274	0.00236	< 0.000001	0.67	0.59	0.82	3.75
33	4540.27	0.01417947900114844	0.75	0.0000295	0.00116	0.645	1.43	1.7	1.95	2.19
38	4939.09	0.01460380606676501	1.46	0.0000295	0.00619	0.297	2.07	2.09	2.73	3.53
39	5004.15	0.152500867738517	19.07	0.0000624	0.00137	< 0.000001	1.94	2.1	20.38	1.32
58	6481.8	0.1413270015769095	2.21	< 0.000001	0.00116	0.000238	2.65	0.49	2.61	2.7
60	6553.06	0.1348150106140109	10.08	0.00000118	0.000449	0.033	8.34	2.15	6.52	12.24
62	6591.84	0.1677498605462907	7.98	< 0.000001	0.000503	< 0.000001	2.65	10.6	2.62	3.36

[a] Peak number: correlative numbering of the peak in the average spectra; Mass: *m/z* value; Weight: relative contribution of each peak to the model; DAve: difference between the maximal and the minimal average peak area/intensity of all classes; PTTA: *p* value of *t*-/ANOVA test; PWKW: *p* value of Wilcoxon/Krustal-Wallis test (preferable for nonnormally distributed data); PAD: *p* value of Anderson-Darling test, which gives information about normal distribution (*p*-value AD ≤ 0.05, nonnormally distributed; *p*-value AD > 0.05, normally distributed); Ave1, Ave2, Ave3 and Ave4, peak area/intensity average of class 1 (CC5), class 2 (CC8), class 3 (CC22) and class 4 (CC398), respectively.

Figure 24 shows the best peaks whose presence or absence is specific for a given CC. For instance, the biomarker peak found at 3,891 *m/z* was only present in the spectra of CC5 strains and absent in all other spectra (see Figure 23). Mass spectra from non-CC5 isolates exhibited a peak at 3,876 *m/z*. A biomarker peak found at 6,592 *m/z* was unique to the spectra of CC8 isolates and peaks at 6,481 and 6,553 *m/z* were always absent in CC8 isolates while present in all other CCs. Specific peaks for CC22 and CC398 at 5,004 and 4,514 *m/z,* respectively, were also identified.

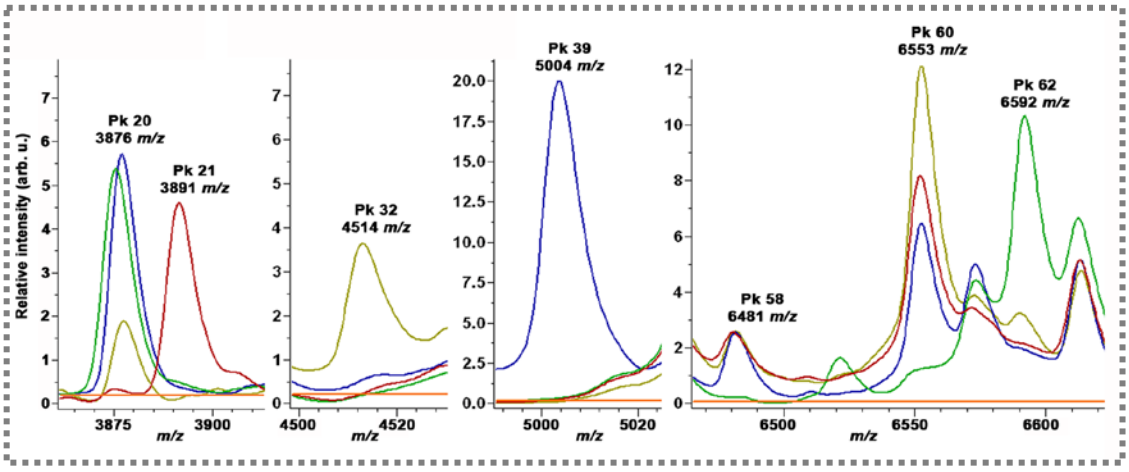


Figure 24: Averaged spectra plots showing the presence or absence of the relevant peak biomarkers for MALDI-TOF MS discrimination of the main 4 MRSA CCs in the SNN model. CC5 (red), CC8 (green), CC22 (blue); CC398 (yellow). *X*-axis shows the mass per charge ratio values (*m/z)* and *y*-axis indicates the intensities of peaks expressed in arbitrary intensity units (from **Figure 1, article 4, Annex I**).

Figure 25 shows the pairwise two-dimensional distribution for the best separating peaks and supports their potential to differentiate isolates belonging to different clonal complexes. For instance, peaks at 3,876 and 3,891 *m/z* clearly discriminate isolates belonging to CC5, peaks at 6,553 and 6,592 *m/z* distinguish isolates from CC8 and peaks at 4,514 and 5,004 *m/z* categorise isolates from CC398 and CC22, respectively.

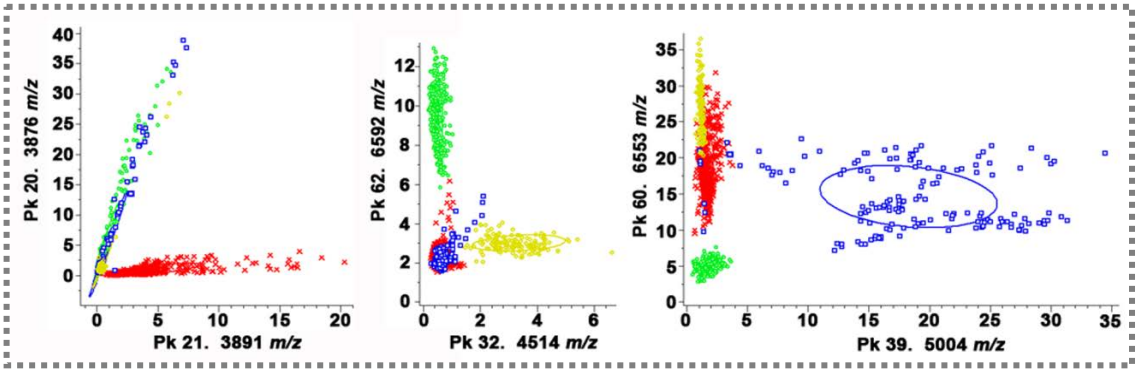


Figure 25: 2D peak distribution diagrams displaying the paired distribution for the best separating peaks in the SNN model; the ellipses represent the standard deviation of the class average of the peak areas/intensities. CC5 (red), CC8 (green), CC22 (blue); CC398 (yellow). The peak numbers and *m/z* values are indicates on the x- and y-axis (from **Figure 2, article 4, Annex I**).

An external validation of the model using the 46 isolates of the validation set was implemented to further assess the performance of the model. Six independent spectra per strain were loaded into the ClinProTools software and classified according to the 11 biomarker peaks selected with the SNN algorithm. All but one replica were correctly classified into their corresponding CC, resulting in a positive predictive value (PPV) of 99.6% (see Table 31).

Table 31: External validation of the supervised neural network model using the ClinProTools and the MALDI BioTyper software (from Table 3, article 4, Annex I).

				Spectra Classification					
Software Used	**CC**	**Nº of Isolates**	**Nº of Spectra[a]**	**CC5**	**CC8**	**CC22**	**CC398**	**Correct classified part of valid spectra (%)**	**PPV (%)**
ClinProTools	5	15	90	90	0	0	0	100	**99.6**
	8	15	90	0	90	0	0	100	
	22	4	24	0	0	23	1	96	
	398	12	72	0	0	0	72	100	
MALDI BioTyper	5	15	90	87	0	2	1	96.7	**98.9**
	8	15	90	0	90	0	0	100	
	22	4	24	0	0	24	0	100	
	398	12	72	0	0	0	72	100	

CC, clonal complex; PPV, positive predictive value

[a] Each isolate was spotted three times and each spot was analysed twice resulting in 6 mass spectra per isolate.

Automated classification of spectra

In order to attempt automated subtyping of MRSA isolates to their corresponding CCs using the MALDI BioTyper software, spectra from each strain within the reference set were used to generate BioTyper Main Spectra (MSP) signatures representative of all 4 CCs that were subsequently incorporated to our local taxonomy database. CC-specific MSPs were then used to create associated subtyping MSPs that were manually edited by resetting the weights of all peaks to 0% except for those peaks identified in the SNN model, that were modified to incorporate the weight values provided by the model (see Table 30).

Spectra from all 46 isolates of the validation set were then analysed with the MALDI BioTyper software against a local database that contained the subtyping MSPs. The best subtyping MSP match was recorded for each spectrum and, with the exception of three spectra from two CC5 isolates that were identified as either CC22 or CC398 (logscore values $\leq$ 2.4), all spectra were correctly classified (logscore values $\geq$ 2.6) resulting in a PPV of 98.9% (see Table 31). In view of these results, an arbitrary cut-off logscore value of 2.6 was chosen to determine the correct subtyping of test spectra.

To test the performance of the subtyping MSPs when faced against isolates belonging to CCs other than those included in the model, spectra from an additional set of 13 isolates representing sporadic MRSA clones (see Table 28) were also analysed with the automated approach. As expected, the MALDI BioTyper aligned the peak profiles of sporadic isolates to the best matching subtyping reference spectrum and, therefore, attempted to classify them into one of the 4 CCs included in the database. The majority of the spectra were

classified as either belonging to CC398 or CC22 with logscore values < 2.6. Five out of the 13 sporadic isolates [CC30 (n=3), CC45 (n=1) and CC80 (n=1)], however, presented at least one spectra with a logscore value ≥ 2.6 (see Table 32), above the arbitrary confidence threshold.

Table 32: Discriminatory power of the supervised neural network model performed in 13 isolates representing the minor MRSA clones circulating in the Hospital Universitari de Bellvitge using MALDI Biotyper software (from **Table 4, article 4, Annex I**).

Isolate	CC	ST	MALDI Biotyper classification (CC)[a]	Score[b]
1	1	1	398	2.597
2	1	1	398	2.498
3	30	30	398	**2.618**
4	30	714	398	2.517
5	30	1870	398	**2.609**
6	30	30	398	2.237
7	30	36	398	**2.613**
8	45	45	398	**2.606**
9	45	1871	398	2.544
10	72	72	398	2.288
11	72	72	398	2.371
12	72	72	22	2.435
13	80	88	398	**2.693**

CC: clonal complex; ST: sequence type. Isolates showing logscore values ≥ 2.6 are highlighted in grey

[a] Only the logscore with the highest value for all spectra originated from the same isolate is shown.

[b] Best score obtained for the subtyping classification of the 6 replicas.

1.3. Investigation of resistance and virulence genetic determinants of MRSA bacteremic clones.

Study 8: Resistance and virulence determinants characterization of endemic MRSA strains analysed by DNA-Microarray Genotyping (DMG).

DGM technology provides genetic information on resistant and virulence determinants allowing a complete characterization of MRSA isolates. The main objective of this study was to apply this new diagnostic microarray technique to genotype the major MRSA clones circulating in the HUB.

DMG was performed on 130 MRSA blood isolates from the 1990-2013 period using the Staphytype kit (Clondiag). The selection of isolates included: CC8 [ST247-SCC*mec*I-*agr*I (n=26); ST8-SCC*mec*IV-*agr*I (n=21); ST1819-SCC*mec*I-*agr*I (n=6)]; CC5 [ST125-SCC*mec*IV-*agr*II (n=36); ST146-SCC*mec*IV-*agr*II (n=13); ST228-SCC*mec*I-*agr*II (n=12); ST5-SCC*mec*IV-*agr*II (n=4)] and CC22 [ST22-SCC*mec*IV-*agr*I (n=12)].

The DNA microarray assigned all the MRSA isolates investigated to the correct CC and *agr* polymorphism. However, SCC*mec* typing was not solved in 13% (17/130) of the studied isolates: two of ST5, four of ST125, nine of ST146, one of ST228 and one of ST8. Another drawback of this technique was the assignation of ST247 to strains belonging ST1819 (a Single Locus Variant of ST247).

Antimicrobial resistance genes detected by DMG were according to phenotypic resistance expression (see Table 33). All 130 MRSA isolates were positive for *mecA* gene and 119 isolates were positive for *blaZ* gene in array hybridization. Macrolide resistance of ST247/ST1819 (32/32), ST8 (4/21) and ST228 (11/12) strains with a MLS_B phenotype was due to the presence of *erm*A

gene while *erm*C gene was the responsible for the MLS_B phenotype in ST125 (6/36), ST146 (5/13), ST8 (1/21) and ST22 (7/12) strains. The MS_B phenotype, which is mediated by *msrA* gene, was observed in strains belonged to ST125 (25/36), ST146 (1/13) and ST8 (5/21) strains.

Aminoglycoside resistance (gentamicin and tobramycin) mediated by the bifunctional modifying enzyme *aacA_aphD* plus *aadD* genes was detected in all strains of ST1819/ST247 (n=32) and in a single isolate of ST8. The remaining aminoglycoside resistance isolates harboured combinations of several genes for aminoglycoside-modifying enzymes such as: *aacA_aphD* plus *aad*A plus *aphA3* in a single isolate of ST8 and two isolates of ST125; *aacA_aphD* plus *aphA3* in isolates belonged to ST228 (n=11/12); *aadD* plus *aphA3* in ST8 (4/21), ST125 (23/36) and ST146 (1/13) isolates; *aacA_aphD* in ST8 (1/21), ST5 (1/4) and ST125 (1/36) isolates; *aphA3* in ST228 (1/12); *aadD* in ST8 (7/21), ST125 (6/36) and ST146 (9/13) isolates.

Tetracycline resistance was mediated by *tetM* in all isolates of ST247/ST1819 and was mediated by *tetK* in one isolate of ST8 and one isolate of ST125. Additionally, cotrimoxazole resistance was detected in two isolates of ST247/ST1819 and harboured the *dfrA* gene. However, this gene was also found in five isolates that were susceptible to cotrimoxazole. Chloramphenicol resistance was only detected in isolates of ST125 (n=7) and is due to the *cat* gene. Finally, the only resistance determinant that was detected in all CCs except in CC22 was chromosomal *fosB* (metallothiol transferase, fosfomycin resistance) (see Table 33).

Table 33: Antibiotic resistance patterns and resistance genes of the 130 MRSA isolates recovered in HUB (1990-2013).

MLST typing	Isolates No.	PHENOTYPE*	Beta-lactams				Macrolides					Aminoglycosides			Cotrimoxazole	Tetracyclines		Cloramphenicols	Fosfomycin
			mecA	*blaZ*	*blaI*	*blaR*	*ermA*	*ermB*	*ermC*	*linA*	*msrA*	*aacA_aphD*	*aadD*	*aphA_3*	*dfrA*	*tetK*	*tetM*	*cat*	*fosB*
247/1819	20/6	OX,E,CC,TE,GM,NN,CIP,RA	+	+(20/5)	+(20/5)	+(20/5)	+	-	-	-	-	+	+	-	-	-	+	-	+
	1/1	OX,E,CC,TE,GM,NN,SXT,CIP,RA	+	+(0/1)	+(0/1)	+(0/1)	+	-	-	-	-	+	+	-	+	-	+	-	+
	4/0	OX,E,CC,GM,NN,TE,CIP	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	-	+
8	1	OX,E,CC,CIP	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+
	3	OX,E,CC,NN,CIP	+	+(1)	+(1)	+(1)	+	-	-	-	-	-	+	-	-	-	-	-	+
	3	OX,E,NN,CIP	+	+	+(2)	+(2)	-	-	-	-	+	-	+	+	-	-	-	-	+
	1	OX,E,CC	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	OX,E,CIP	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+
	1	OX,E,GM,NN,CIP	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	+
	5	OX,NN,CIP	+	+(4)	+(4)	+(4)	-	-	-	-	+(1)	-	+	+(1)	-	-	-	-	+
	1	OX,TE	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+
	3	OX,CIP	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
	2	OX,GM,NN,CIP	+	+	+	+	-	-	-	-	-	+	+(1)	-	+	-	-	-	+
228	10	OX,E,CC,GM,NN,CIP,RA	+	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+
	1	OX,E,CC,GM,NN,CIP	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-
	1	OX,GM,NN,CIP,RA	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	-	+
5	3	OX,CIP	+	+(2)	+(2)	+(2)	-	-	-	-	-	-	-	-	-	-	-	-	+
	1	OX,GM,NN,CIP	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+
125	2	OX,E,CC,NN,CIP	+	+(1)	+(1)	+(1)	-	-	+	-	+(1)	-	+	+(1)	-	-	-	-	+
	3	OX,E,CC,GM,NN,CIP	+	+(2)	+(2)	+(2)	-	-	+(2)	-	+(2)	+	+	+(2)	+(2)	-	-	-	+
	1	OX,E,C,CIP	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	+	+
	1	OX,E,CC,C,GM,NN,CIP	+	+	+	+	-	-	+	-	+	+	+	+	-	-	-	+	+
	1	OX,E,CC,C,NN,CIP	+	+	+	+	-	-	+	-	+	-	+	+	-	-	-	+	+
	2	OX,E,CIP	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+
	4	OX,E,C,NN,CIP	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	+	+
	1	OX,E,NN,CIP,RA	+	+	+	+	-	-	-	-	+	-	+	+	-	-	-	-	+
	1	OX,E,TE,NN,CIP	+	+	+	+	-	-	-	-	+	-	+	+	-	+	-	-	+
	14	OX,E,NN,CIP	+	+	+	+	-	-	-	-	+	-	+	+(13)	-	-	-	-	+
	2	OX,E,GM,NN,CIP	+	+	+	+	-	-	-	-	+	+	+	+(1)	+(1)	-	-	-	+
	3	OX,NN,CIP	+	+(1)	+(1)	+(1)	-	-	-	-	-	-	+	-	-	-	-	-	+
	1	OX,CIP	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
146	3	OX,E,CC,NN,CIP	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	+
	6	OX,NN,CIP	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+
	1	OX,CIP	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+
	2	OX,E,CC,CIP	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+
	1	OX,E,NN,CIP	+	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	+
22	1	OX,E,CC	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
	6	OX,E,CC,CIP	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
	5	OX,CIP	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	130																		

* Oxacillin (OX), Erythromycin (E), Clindamycin (CC), Tetracycline (TE), Gentamicin (GM), Tobramycin (NN), Ciprofloxacin (CIP), Rifampicin (RA), Trimethoprim-sulphamethoxazole (SXT), Chloramphenicol (C), Quinupristin-dalfopristin (SYN).

Numbers in parentheses indicate the number of strains sharing the same characteristic.

Regarding virulence genes, one isolate of ST125 presented the *tsst*-1 gene that encodes Toxic Shock Sindrome Toxin (TSST-1). Exfoliative toxins *et*A, *et*B were detected in one isolate of ST228. Three ST8 strains were positive for Panton-Valentine leucocidin (PVL). Enterotoxins profiles differed between the two main clones: ST247 carried *ent*A gene while ST125 presented *ent*G,I, M, N, O and U genes (see Table 34). All MRSA strains produce superantigen-like toxins, called *ssl*/*set*, but different ST groups were encoded by different allelic variants (see Table 35). Strains belonging to ST22 (CC22) did not exhibited *ssl*03, *ssl*06, *ssl*08 and *ssl*11 genes and strains of CC5 did not show the *ssl*06 gene. Only CC8 strains presented *ssl06* genes. Moreover, strains of CC8 and CC5 presented the same allelic profile of superantigens *ssl*01-02-03-04-07-08 genes.

Concerning capsule and biofilm genes, all tested isolates belonged to capsule type 5 and have the same biofilm genes profile: positive for *icaA*, *icaC* and *icaD* genes, encoding the intercellular adhesion proteins, and negative for *bap* gene, encoding a surface protein involved in biofilm formation (see Table 36). Most microbial surface components recognizing adhesive matrix molecules (MSCRAMM) genes were widely distributed and occurred in nearly all clonal groups. Several genes, like *ebh* (cell wall associated fibronectin-binding protein), *fib* (fibrinogen binding protein) and *fnbB* (fibronectin-binding protein B), were not detectable in ST22 (CC22) (see Table 36). The *cna* (encoding collagen adhesion) occurred only in ST22 (CC22). Some isolates showed deletions of MSCRAMM genes. For instance, *bbp* (bone sialoprotein-binding protein), *cflA* (clumping factor A) and *fnbB* were usually present in CC5.

However, all isolates of the ST146 (CC5) and one isolate of ST5 (CC5) lacked *bbp* gene. Only three isolates of ST228 (CC5) showed *fnbB* gene.

Table 34: Enterotoxin profiles exhibited by each clone circulating in HUB (1990-2013).

agr/CC	MLST	*entA*	*entA* var3	*entC*	*entD*	*entG*	*entH*	*entJ*	*entI*	*entL*	*entM*	*entN*	*entO*	*entU*	Isolates No.
agrII/CC5	5	-	+(3)	-	+(3)	+	-	+(3)	+	-	+	+	+	+	4
agrII/CC5	125	+(1)	+(25)	-	-	+	-	-	+	-	+	+	+	+	36
agrII/CC5	146	-	-	-	+(2)	+	-	-	+	-	+	+	+	+	13
agrII/CC5	228	+	-	-	-	+(1)	-	-	+(1)	-	+(1)	+(1)	+	+(1)	12
agrI/CC8	1819/247	+(31)	-	-	-	-	-	-	-	-	-	-	-	-	32
agrI/CC8	8	+(15)	-	-	+(8)	-	-	+(7)	-	-	-	-	+(1)	-	21
agrI/CC22	22	-	-	+(3)	-	+	-	-	+	+(3)	+	+	+	+	12
Total No.															130

Numbers in parentheses indicate the number of strains sharing the same characteristic.

Table 35: Superantigen profiles (*ssl/set* genes) exhibited by each clone circulating in HUB (1990-2013).

agr/CC	MLST	set6-var1_11	set6-var2_11	set6-var1_12	set6-var4_11	ssl1 / set6 (COL)	ssl1 / set6(Mu50/N315)	ssl1 / set6 (MRSA252)	ssl1 / set6_other variants	ssl2 / set7	ssl2 / set7 (MRSA252)	ssl3 / set8	ssl03	ssl4 / set9	ssl4 / set9 (MRSA252, SAR0425)	ssl5 / set3	ssl05	ssl5 / set3 (MRSA252)	ssl6 / set21	ssl06-NCTC+MW2	ssl7 / set1	ssl7 / set1 (AF188836)	ssl8 / set12	ssl08	ssl11 / set2 (COL)	ssl11 / set2(Mu50/N315)	Isolates No.
agrII/CC5	5	+	-	+	+(1)	-	+	-	-	+	-	+	+	+	-	+	+	-	-	-	+	-	+	+	-	+	4
agrII/CC5	125	+	-	+	+(11)	-	+	-	-	+	-	+	+	+	-	+	+	-	-	-	+	-	+	+	-	+	36
agrII/CC5	146	+	-	+	+(4)	-	+	-	-	+	-	+	+	+	-	+	+	-	-	-	+	-	+	+	-	+	13
agrII/CC5	228	+	-	+	+(2)	-	+	-	-	+	-	+	+(6)	+	-	+	+	-	-	-	+	-	+	+	-	+	12
agrI/CC8	1819/247	+	-	+(5)	+	+	-	-	-	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+	-	32
agrI/CC8	8	+	-	+(2)	+	+	-	-	-	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+	-	21
agrI/CC22	22	-	+	+(8)	-	-	-	+(8)	+(4)	+(1)	+(11)	-	-	-	+	+(10)	-	+	-	-	-	+	-	-	-	-	12
Total																											130

Numbers in parentheses indicate the number of strains sharing the same characteristic.

Table 36: Biofilm and MSCRAMMs genes revealed by each clone circulating in HUB (1990-2013).

agr/CC	MLST	Biofilm genes [a]				MSCRAMMs genes [b]														Isolates No.
		icaA	*icaC*	*icaD*	*bap*	*bbp*	*clfA*	*clfB*	*cna*	*ebh*	*eno*	*fib*	*ebpS*	*fnbA*	*fnbB*	*map*	*sdrC*	*sdrD*	*vwb*	*sasG*	
agrII/CC5	5	+	+	+	-	+(3)	+	+	-	+	+	+	+	+	+	+	+	+	+	+	4
agrII/CC5	125	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+(34)	+	+	36
agrII/CC5	146	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+(7)	+	+(11)	13
agrII/CC5	228	+	+	+	-	+	+	+	-	+	+	+	+	+	+(3)	+	+	+	+	+	12
agrI/CC8	1819/247	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	32
agrI/CC8	8	+	+	+	-	+(17)	+	+	-	+	+	+	+	+	+	+(19)	+	+(17)	+	+	21
agrI/CC22	22	+	+	+	-	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+	12
Total No.																					130

[a] *icaA*, intercellular adhesion protein A (N-glycosyltransferase); *icaC*, intercellular adhesion protein C; *icaD*, biofilm PIA synthesis protein D; *bap*, surface protein involved in biofilm formation.

[b] MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; *bbp*, bone sialoprotein-binding protein; *clfA*, clumping factor A; *clfB*, clumping factor B; *cna*, collagen-binding adhesin; *ebh*, cell wall associated fibronectin-binding protein; *eno*, enolase - phosphopyruvate hydratase; *fib*, fibrinogen binding protein; *ebpS*, cell wall associated fibronectin-binding protein; *fnbA*, fibronectin-binding protein A; *fnbB*, fibronectin-binding protein B; *map*, major histocompatibility complex class II analog protein; *sdrC*, Ser-Asp rich fibrinogen-binding - bone sialoprotein-binding protein C; *sdrD*, Ser-Asp rich fibrinogen-binding - bone sialoprotein-binding protein D; *vwb*, Willebrand factor binding protein; *sasG, S. aureus* surface protein G.

Numbers in parentheses indicate the number of strains sharing the same characteristic.

2. Phenotypic and genotypic analysis of the MRSA bacteremic isolates from patients admitted to Spanish hospitals from 2008 to 2009.

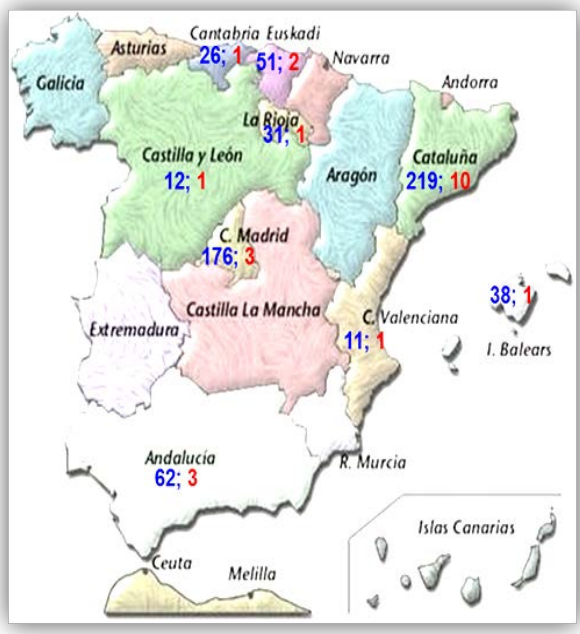
*Additional clinical research information is attached in the papers referred in **Annex II***

These studies were conducted through a multicentre project assisted by the Spanish Network for Infectious Diseases Research (REIPI).Twenty-three Spanish hospitals sent MRSA strains causing bacteremia to the Microbiology Department at HUB, which centralized the microbiology studies.

2.1. Resistance to methicillin and antimicrobial resistance patterns among *S. aureus* strains isolated in different Spanish hospitals.

A total of 610 bacteremic patients were prospectively followed in 23 Spanish hospitals from June-2008 to December-2009 and 626 MRSA isolates were sent for study to the Microbiology Department at the HUB. (see Figure 26).

a)



b)

Hospital	City	MRSA (%) in 2009
HUB	Barcelona	28
General Yagüe	Burgos	35
Univ. Son Dureta	Palma de Mallorca	31
Univ. Ramón y Cajal	Madrid	27
Univ. 12 de Octubre	Madrid	28
Univ. Joan XXIII	Tarragona	25
Univ. Virgen Marcarena	Sevilla	17
Univ. Vall d'Hebron	Barcelona	20
San Pedro de la Rioja	Logroño	23
Parc Tauli	Sabadell	20
Univ. Virgen del Rocio	Sevilla	22
de Cruces	Vizcaya	30
Univ. Mútua de Terrassa	Terrassa	21
Donostia	San Sebastián	18

Figure 26: **a)** Distribution of the 23 Spanish hospitals participating in the study (in red) and isolates number sent to our setting (in blue). **b)** MRSA rate in 2009 of some hospitals participants.

Antibiotic susceptibility testing was performed in all isolates and the global resistance percentages of MRSA clinical isolates to antimicrobial agents is shown in Figure 26. The antibiotic resistance percentages were as follows: erythromycin 62%, clindamycin 28%, gentamicin 19%, tobramycin 66%, ciprofloxacin 95%, rifampicin 3%, tetracycline 3% and mupirocin 20% (see Figure 27).

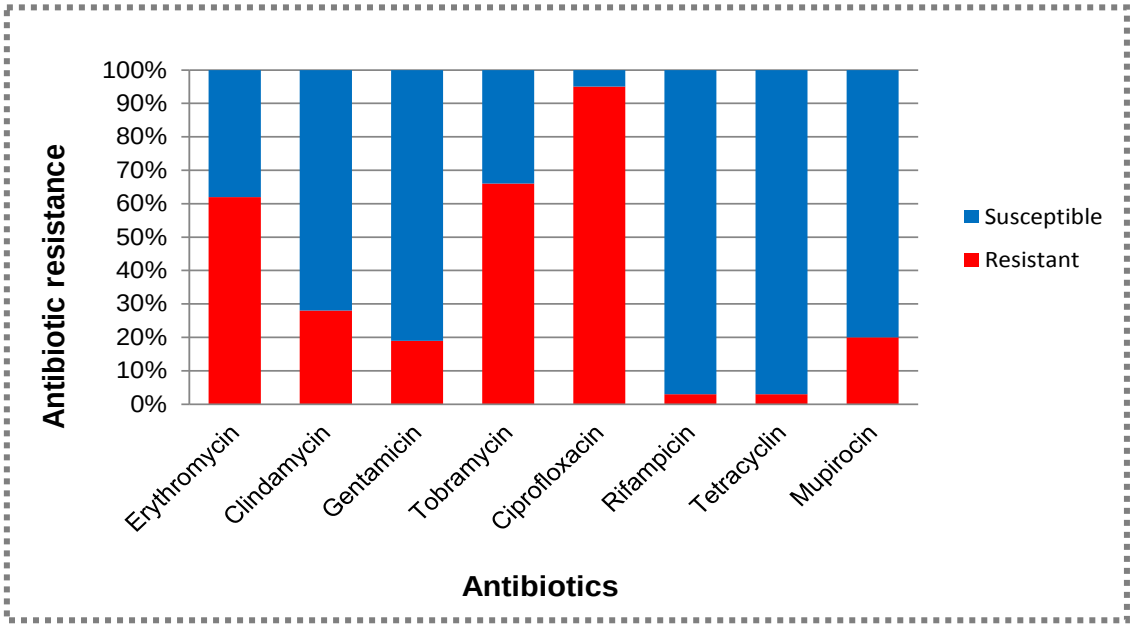


Figure 27: Percentage of antibiotic resistance among 626 MRSA blood strains isolated in 23 Spanish hospitals (2008-2009).

Among isolates resistant to erythromycin: 34% (212/626) showed the MS_B phenotype, 19% (121/626) showed the constitutive MLS_B phenotype and 9% (55/626) showed the inducible MLS_B phenotype.

The 626 MRSA strains were grouped in 31 antibiotic resistance patterns (RP). Figure 28 shows the most frequent RPs of the MRSA strains isolated in Spain (2008-2009). The most frequent combination of resistances were:

erythromycin, tobramycin plus ciprofloxacin (124/626; 20%), ciprofloxacin (97/626; 15%) and tobramycin plus ciprofloxacin (96/626;15%) (see Figure 28).

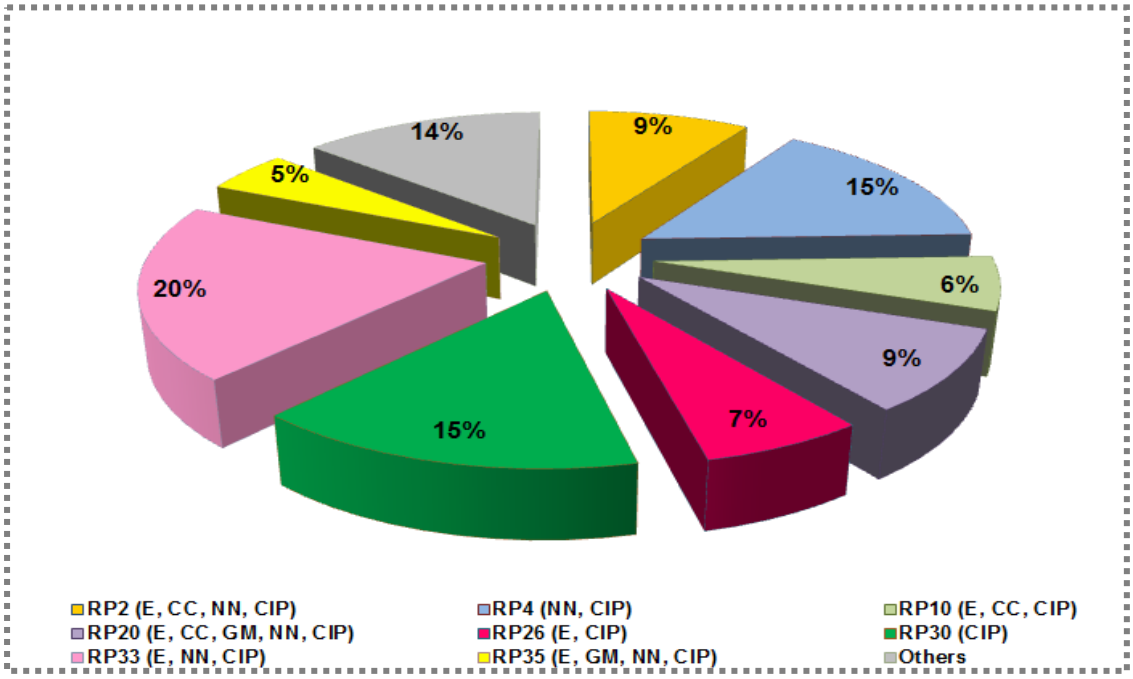


Figure 28: Resistance antibiotic profiles among 626 MRSA blood strains isolated in 23 Spanish hospitals (2008-2009). (E, erythromycin; CC, clindamycin; NN, tobramycin; CIP, ciprofloxacin; GM, gentamicin)

Intermediate-level vancomycin resistance was observed in three isolates (MIC=3mg/L) by broth microdilution method (BMD) and in six (MIC=3mg/L, n=5; MIC=4mg/L, n=1) by Etest method. The Etest provided vancomycin MIC results that were consistently higher than those provided by the reference BMD method. Resistance to daptomycin was observed in three isolates by BMD (MIC=2mg/L), only recognized in one isolate using the Etest method (MIC=1.5mg/L).

Vancomycin, daptomycin and linezolid MIC_{90} values by microdilution were 1, ≤1 and ≤2mg/L respectively (see Tables 37 A, B and C).

Table 37: CMI values for A) VA (vancomycin); B) DA (daptomycin); C) LZD (linezolid), among 626 MRSA blood isolates identified in 23 Spanish hospitals (2008-2009). *Mdil, microdilution.

A)

MIC (mg/L)	VA	
	Etest (n)	Mdil* (n)
≤ 0.5	50	84
0.75	62	350
1	250	153
1.5	199	33
2	59	3
3	5	3
4	1	0
Total (n)	626	626
MIC_{50}	1	0.75
MIC_{90}	1.5	1

B)

MIC (mg/L)	DA	
	Etest (n)	Mdil* (n)
≤1	625	623
1.5	1	-
2	0	3
Total (n)	626	626
MIC_{50}	≤1	≤1
MIC_{90}	≤1	≤1

C)

MIC (mg/L)	LZD
	Mdil* (n)
≤ 2	617
4	8
> 4	1
Total (n)	626
MIC_{50}	≤ 2
MIC_{90}	≤ 2

2.2. Molecular characteristics of MRSA clones causing bloodstream infections using genotypic techniques.

To describe the molecular characteristics of the Spanish MRSA population, PFGE, *agr* type and PVL genes determination was performed in all 626 bacteremic isolates. Out of this collection a selection of 391 (391/626; 63%) isolates, including all genetic variants detected by PFGE, was characterized by SCC*mec*, MLST, and *spa* typing. Of note, in the remaining 235 isolates, genetic background classification was inferred using the PFGE genotype.

Pulsed-field gel electrophoresis (PFGE) characterization

Thirty PFGE types were detected, with types PFGE-2 (413/626; 66%) and PFGE-4 (57/626; 9%) accounting 75% (470/626) of the strains. The third most prevalent PFGE type was PFGE-12 (43/626; 7%) followed by PFGE-5 (35/626; 6%). The remaining 78 (12%) isolates belonged to sporadic clones (27 different PFGE types) that were mostly represented by less than eight isolates and included the majority of community-acquired clones (see Figure 30).

Staphylococcal cassette chromosome *mec* (SCC*mec*) types

SCC*mec* element was investigated in a selection of 391 MRSA blood isolates. The distribution of the different SCC*mec* types among the major genotypes is shown in Figure 28. SCC*mec* type IV accounted for 91% (355/391) of the isolates and was detected in three major PFGE types (PFGE-2, PFGE-4 and PFGE-12) and 12 minor clones. SCC*mec* type I was identified in 21 isolates and was associated with one of the major genotypes (PFGE-5) and two minor clones. SCC*mec*II was found in five isolates of two sporadic clones. SCC*mec* type V was only found in nine isolates that belonged to nontypable (NT)-PFGE clone. Finally, one isolate could not be typed by the SCC*mec* typing scheme used in this study.

Multilocus sequence typing (MLST)

Molecular typing by MLST of 391 isolates showed that isolates of type PFGE-2 were CC5 (ST5, ST125, ST146-SCC*mec*IV) and isolates of PFGE-4 were CC22 (ST22, ST217-SCC*mec*IV) accounting 75% of all studied isolates. Moreover, PFGE-12 corresponded to clone ST8-SCC*mec*IV and PFGE-5 belonged to clone ST228-SCC*mec*I, a double locus variant (DLV) of ST5 and ST125 (see Figure 30). Twelve percent of the isolates were associated to 26 different PFGE types and corresponding to minor clones such as ST72-SCC*mec*IV (a triple locus variant, TLV of ST8), ST931-SCC*mec*IV, ST398-SCC*mec*IV/V, ST30-SCC*mec*II/IV, ST36-SCC*mec*II, ST714-SCC*mec*IV, ST1870-SCC*mec*IV, ST1870-SCC*mec*II, ST1-SCC*mec*IV, ST45-SCC*mec*IV, ST1871-SCC*mec*IV and ST88-SCC*mec*II (see Figure 30 and Table 39).

To plot the distribution of each ST in different autonomous communities of Spain, we constructed a tree using PHILOViZ software (see Figure 29). For instance, ST125, ST146 and ST8 could be detected in most of the Spanish autonomous communities. In contrast, ST5, ST45, ST435, ST30 and ST217 were only found in Cataluña.

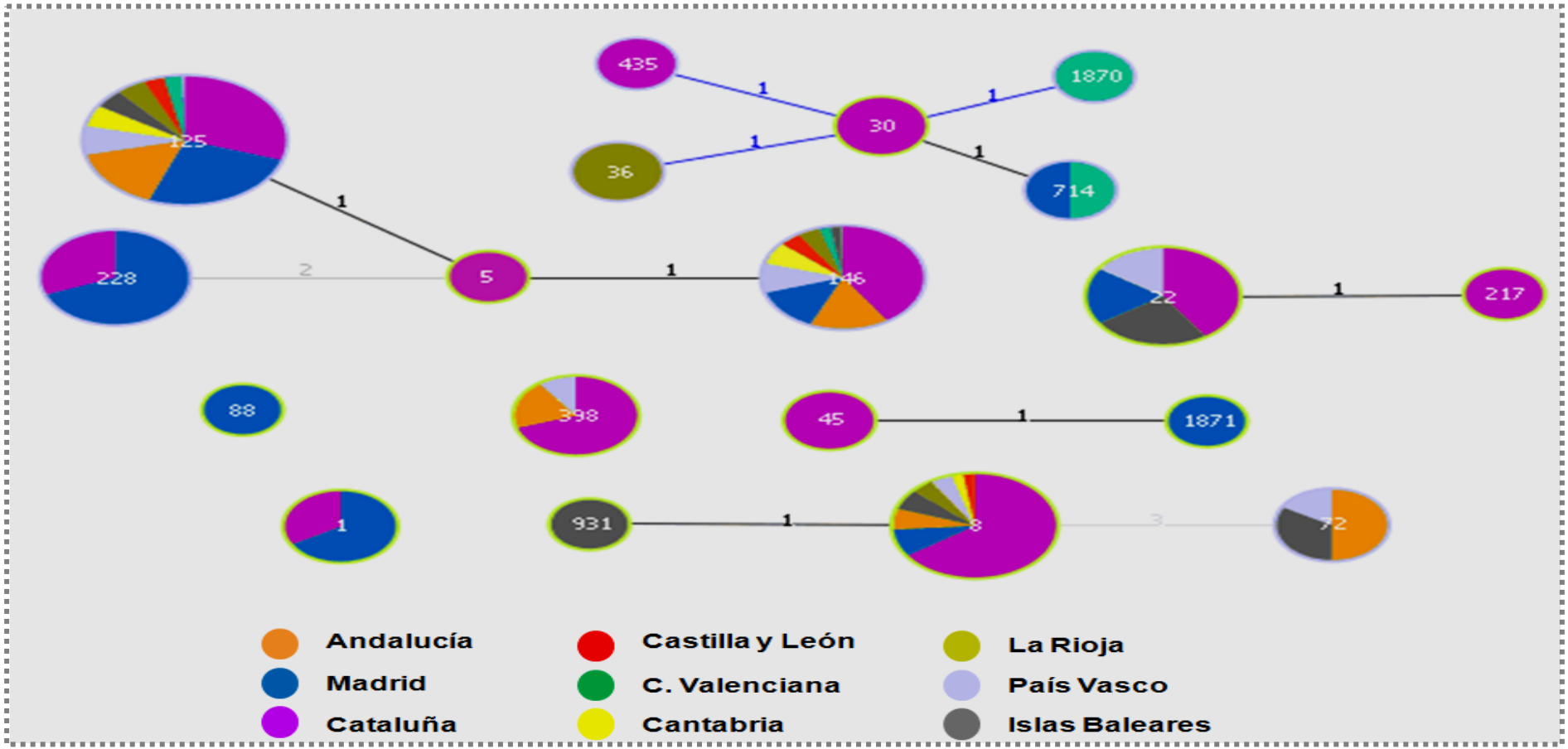


Figure 29: goeBURST diagram of the relationships between 391 MRSA isolates recovered from bloodstream infections in distinct Autonomous Communities of Spain. The size of each circle is proportional to the number of isolates with that particular ST in a logarithmic scale. Coloring depends on geographical origin of isolates. STs assigned to the same CC at TLV level are linked by straight lines: black lines, link drawn without recourse to tiebreak rules; blue lines, link drawn using tiebreak rule 1 (number of SLVs); gray lines, link drawn at DLV (darker gray) or TLV (lighter gray). Putative CC founders are identified by a light green line.

Accesory gene regulator (*agr*) types

agr group was investigated in all isolates (n=626) included in this study. The most frequent *agr* type was type II (472/626; 75%), which grouped strains belonging to the major clones CC5 (ST5, ST125, ST146-SCC*mec*IV) and ST228-SCC*mec*I and 17 sporadic clones. *agr* type I was present in 23 % (141/626) of the strains belonging to the major clones ST22, ST217-SCC*mec*IV and ST8-SCC*mec*IV and 11 sporadic clones including community clones such ST398-MRSA pig-associated clone and USA300 or USA300-like variants. Only 2% (13/626) of the MRSA strains harboured *agr* type III; these strains belonged to clones ST1-SCC*mec*IV (n=4), ST30-SCC*mec*II (n=1), ST30-SCC*mec*IV (n=2), ST88-SCC*mec*II (n=1), ST36-SCC*mec*II (n=1), ST1870-SCC*mec*-II (n=1) and ST714-SCC*mec*IV (n=2). None of the MRSA strains presented *agr* type IV.

Panton-Valentine leukocidine (PVL) genes and arginine catabolic mobile element (ACME).

PVL was found to be positive in 20 isolates; 8 of them belonged to the USA300 genotype (ST8-SC*Cmec*IV-*agr*I), 7 related to the USA300/ACME-negative (ST8-SCC*mec*IV-*agr*I), 2 related to non USA300 genotype (ST8-SCC*mec*IV-*agr*I), 2 to PFGE-2 (ST125-SCC*mec*IV-*agr*II) and one related to the CA-MRSA clone ST30-SCC*mec*IV-*agr*III (see Figure 30).

Molecular features and antibiotic resistance patterns (RP) relationship.

Overall, molecular features of MRSA strains isolated in Spain from 2008 to 2010 collection are summarized in Figure 30.

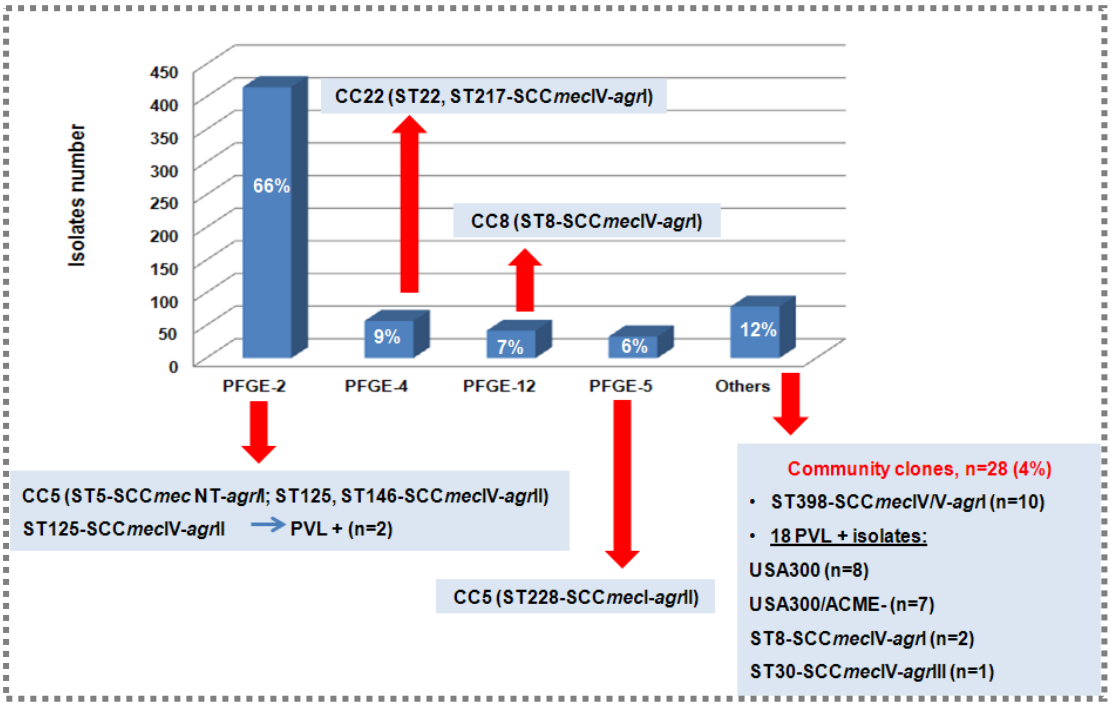


Figure 30: Molecular characterization of 626 MRSA blood strains identified in 23 Spanish hospitals (2008-2009).

Additional molecular and resistance traits of MRSA clones circulating in Spain during the 2008-2009 period are summarized in Table 38. In general, PFGE-2 was mainly associated (92%; 381/413) with eight different RPs (RP2, RP4, RP10, RP20, RP26, RP30, RP33 and RP35). In contrast, PFGE-4 was mostly characterized by two non-multiresistant profiles such as RP10 and RP30 (95%; 54/57). All strains that belonged to PFGE-5 were associated with the multiresistant profile RP20. PFGE-8 was associated with a wide diversity of RPs (see Table 38).

Table 38: Molecular features and resistance patterns of 626 MRSA blood strains isolated in Spain during the 2008-2009 period.

RP	Strains n	ST5/ST125/ST146 SCC*mec*IV;*agr*II	ST22/ST217 SCC*mec*IV;*agr*I	ST8 SCC*mec*IV;*agr*I	ST228 SCC*mec*I;*agr*I	Others
		PFGE-2	PFGE-4	PFGE-12	PFGE-5	
2	57	43	0	6	0	8
3	1	0	0	0	0	1
4	96	73	0	12	0	11
9	18	9	1	5	0	3
10	38	12	23	1	0	3
12	18	9	0	0	0	8
13	2	2	0	0	0	0
15	1	1	0	0	0	0
20	58	12	1	1	35	9
22	1	1	0	0	0	0
23	1	0	0	0	0	1
24	1	1	0	0	0	0
26	41	36	0	1	0	4
27	5	0	0	2	0	3
30	97	61	31	3	0	2
33	124	115	0	9	0	0
35	30	29	1	0	0	0
37	2	1	0	0	0	1
38	1	0	0	1	0	0
41	1	0	0	0	0	1
42	5	1	0	1	0	3
45	2	2	0	0	0	0
48	8	0	0	0	0	8
49	1	1	0	0	0	0
53	1	0	0	0	0	1
56	1	1	0	0	0	0
65	4	3	0	1	0	0
69	4	0	0	0	0	4
71	4	0	0	0	0	4
74	2	0	0	0	0	2
79	1	0	0	0	0	1
Total	**626**	**413**	**57**	**43**	**35**	**78**

RP2 (E, CC, NN, CIP);
RP3 (E, CC, NN);
RP4 (NN, CIP);
RP9 (G, NN, CIP);
RP10 (E, CC, CIP);
RP12 (beta-lactams);
RP13 (E, CC, C, NN, CIP);
RP15 (C, NN, CIP);
RP20 (E, CC, G, NN, CIP);
RP22 (G, NN);
RP23 (E, CC, C, G, NN, CIP);
RP24 (C, NN);
RP26 (E, CIP);
RP27 (NN);
RP30 (CIP);
RP33 (E, NN, CIP);
RP35 (E, G, NN, CIP);

RP37 (E, CC);
RP38 (E, C, NN, CIP);
RP41 (NN, SXT, CIP, RA)
RP42 (TE);
RP45 (E, CC, NN, CIP RA)
RP48 (E, CC, G, NN, CIP, RA);
RP49 (E, NN, TE, CIP);
RP53 (E);
RP56 (NN, TE, CIP);
RP65 (E, NN, CIP, RA);
RP69 (E, CC, TE);
RP71 (TE, CIP);
RP74 (E, CC, NN, TE, CIP);
RP79 (E, CC, G, NN, TE)

E=Erythromycin; **CC**=Clindamycin;
NN=Tobramycin; **CIP**=Ciprofloxacin;
G=Gentamicin; **C**=Chloranphenicol;
TE=Tetracycline; **RA**=Rifampicin;
SXT=Trimethoprim-sulphamethoxazole

***S. aureus* protein A (*spa*) typing**

A collection of 391 isolates was selected to evaluate the distribution and frequencies of *spa* types including all PFGE types and subtypes and all the antibiotic resistance profiles identified in each hospital.

Among the 391 studied isolates between 2008 and 2009, 365 (93.4%) clustered into four main *spa*-CCs (55 *spa* types), 19 (4.9%) were singletons (5 *spa* types), five (1.3%) were excluded (2 *spa* types) from the BURP analysis and two (0.5%) were not typable. The main *spa*-CC found was *spa*-CC686/002 (36 *spa* types) which included 270 strains, associated with CC5 (ST5, ST125, ST146). Two predominant types, t067 and t002, were detected in *spa*-CC686/002, accounting for 42% (163/391) and 11% (43/391) respectively of all selected isolates. *spa* type t067 was the only type identified in all hospitals. However, t002 and t008 have also been found in many Spanish hospitals. *spa*-CC008, the second *spa*-CC, included 45 strains with 6 different *spa* types and belonged to CC8 (ST8 and ST931). The most common *spa* type was t008 (38/45). In this group, 15 (33%) isolates were PVL positive, showing the PFGE patterns of the USA300 and the USA300-like (ACME negative). On the third position, was *spa*-CC032 (34/391) associated with CC22 (ST22 and ST217) which contained 34 strains and 71% (24/34) of them were *spa*-type t032. Finally, *spa*-CC012/1197 harboured 16 strains (8 different *spa* types), was associated with CC30 (ST30, ST36, ST435, ST714, ST1870) and CC398 (ST398) (see Figure 31). Of note *spa* types t109 (n=13) and t041 (n=6) were only detected in the autonomous communities of Madrid and Cataluña respectively (see Figure 32).

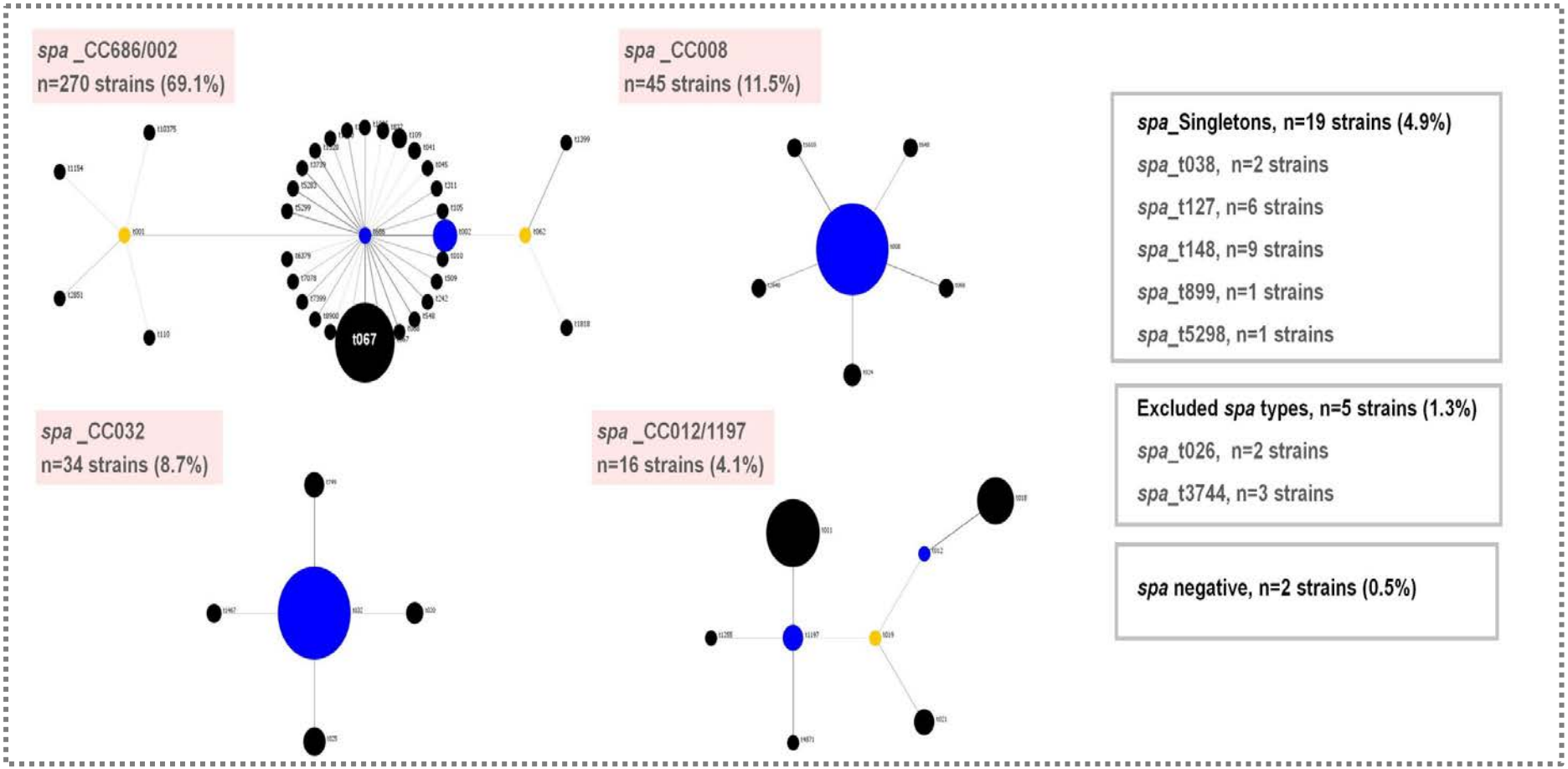


Figure 31: BURP analysis of *spa* data obtained for all 391 strains isolates collected in Spanish hospitals (2008-2009). *spa* types are represented by dots and the related *spa* types are linked by a line. The shade of the line varies according to the evolutionary cost between the *spa* types, where a darker shade represents a lower evolutionary cost. The diameter of the dot is proportional to the quantity of the corresponding *spa* type. The ancestor of *spa*-CC cluster is represented in blue dots while yellow dots show a co-founder.

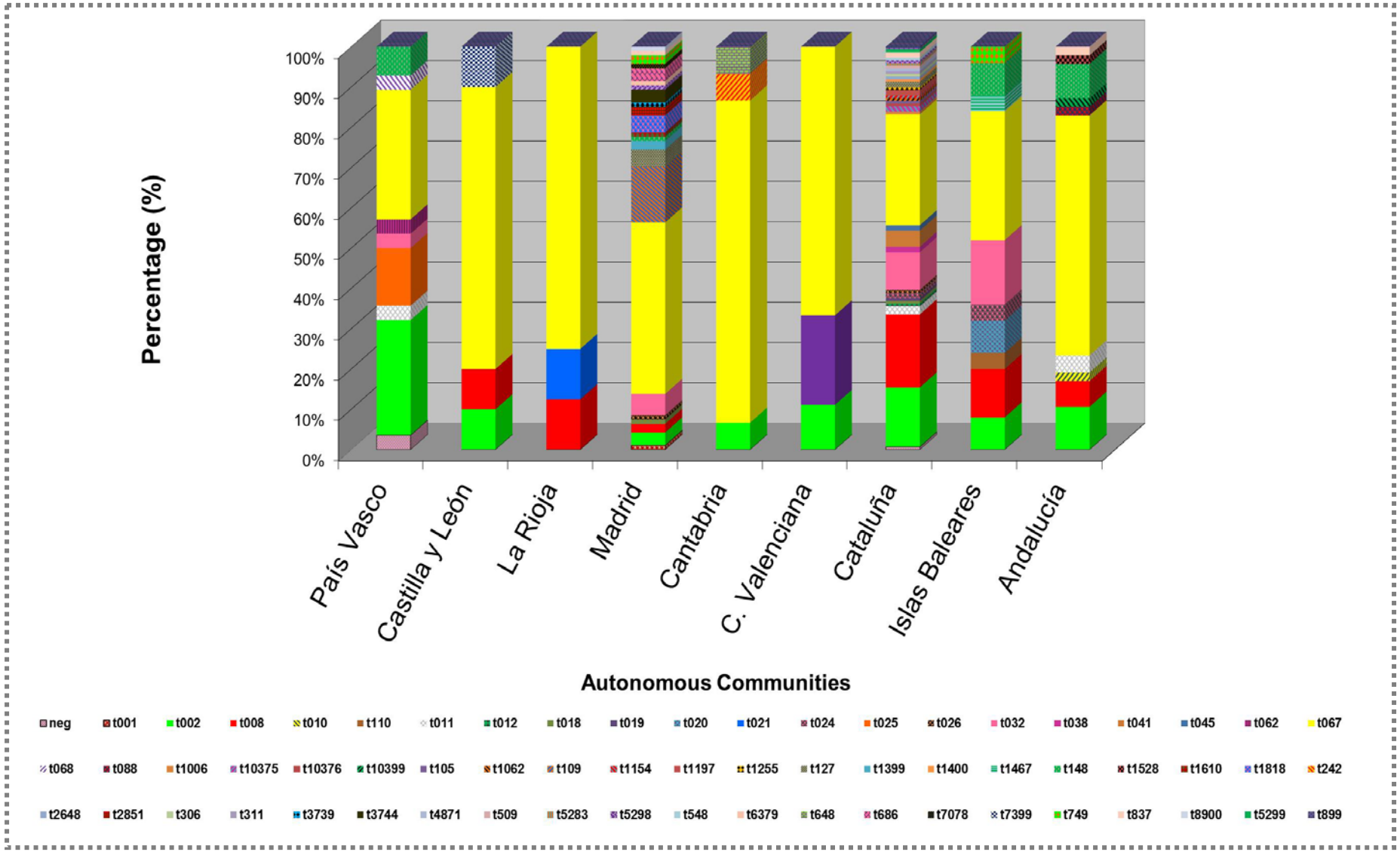


Figure 32: Distribution of *spa* types in the Autonomous Communities of Spain.

Finally, clonal composition of MRSA isolates that are circulating in Spain from 2008 to 2009 are summarized in Table 39.

Table 39: Clonal composition of MRSA strains isolated in Spanish hospitals from 2008 to 2009.

CC (%)	ST	SCC*mec*	*agr*	*spa*	PVL (nº of isolates)	ACME (nº of isolates)
5 (75.24)	5	Nt	II	Nt	-	-
	125	IV	II	Nt, t002, t010, t045, t062, t067, t088, t105, t109, t110, t242, t306, t311, t509, t548, t686, t837, t1006, t1062, t1154, t1399, t1400, t1528, t1818, t2851, t3739, t3744, t5283, t5299, t6379, t7038, t7399, t8900, t10376, t10399	+ (2)	-
	146	IV	II	t002, t010, t045, t062, t067, t088, t105, t109, t110, t242, t306, t311, t509, t548, t686, t837, t1006, t1062, t1154, t1399, t1400, t1528, t1818, t2851, t3739, t3744, t5283, t5299, t6379, t7038, t7399, t8900, t10376, t10399	-	-
	228	I	II	t001, t041, t109, t2851, t3744, t10375	-	-
8 (11.50)	8	IV	I	t008, t024, t068, t148, t648, t1610, t2648	+ (17)	+ (8)
	931	IV	I	t008	-	-
	72	IV	I	t008, t148	-	-
22 (9.11)	22	IV	I	t020, t025, t032, t749, t1467	-	-
	217	IV	I	t032	-	-
398 (1.6)	398	IV/V	I	t011, t026, t899, t1197, t1255, t4871	-	-
30 (1.12)	30	II/IV	III	t012, t019	+ (1)	-
	36	II	III	t021		
	714	IV	III	t018		
	1870	II	III	t018		
1 (0.80)	1	IV	III	t127	-	-
45 (0.48)	45	IV	I	t038	-	-
	1871	IV	I	t026		
88 (0.16)	88	II	III	t5298	-	-

CC, clonal complex; ST, sequence type; SCC*mec*, staphylococcal cassette chromosome *mec*; *agr*, accessory gene regulator; *spa*, staphylococcal protein A; PVL, leukocidin Panton-Valentine; ACME, arginine catabolic mobile element.

* NT, non-typable

Numbers in parentheses indicate the number of strains sharing the same characteristic.

Chapter V

DISCUSSION

The aim of the studies presented in this Thesis was to characterise and define the microbiology features of different MRSA clones present not only in our clinical setting (Hospital Universitari de Bellvitge, HUB) but also in other Spanish hospitals. This microbiologic approach included the following aspects: (i) rate of resistance to methicillin among *S. aureus* clinical isolates detected in HUB and other Spanish hospitals; (ii) antimicrobial susceptibility and activity of therapeutic agents against MRSA clinical isolates; (iii) genetic background of MRSA clones circulating in the HUB and in other Spanish hospitals; and (iv) clonal evolution and geographic spread of MRSA in national environments. The phenotypic and genotypic methods used for the characterization of MRSA isolates allowed us to analyse the value of each technique based on the type of study and type of question to be answered.

The novel automated approach of MRSA categorization into different CC by MALDI-TOF/MS constituted a major contribution on the continuous updating and validation of typing methods required for the characterization of *S. aureus* populations. Additionally, this new typing methodology could easily be implemented in routine laboratories and has a great potential as an epidemiologic tool in MRSA infection control.

1. Epidemiology of endemic MRSA observed in the HUB over the last 25 years (1990-2014). Epidemiology of MRSA in Spanish hospitals (2008-2009).

MRSA is one of the most frequently identified antimicrobial drug-resistant pathogens worldwide and has evolved in a relatively few lineages. Enright and colleagues revealed that some lineages are ecologically highly successful and that most isolates belong to pandemic clones [151]. The multi-resistant nature of most MRSA clones found in hospitals represents a therapeutical challenge for treating serious MRSA infections.

1.1. Evolution of methicillin resistance in *S. aureus* strains and MRSA's antimicrobial susceptibility to old and new anti-staphylococcal agents.

In this study, we report the evolution of MRSA in the HUB during the 1990-2014 period. Following the high rate achieved in 1992, when 34% of all *S. aureus* isolated in the HUB were methicillin resistant, we have observed a steadily increase in the rate of methicillin resistance from 1996 (5%) to 2004 (25%). Since 2004 to 2014, the rate remained stable around 24%. Although not explicitly reported in the literature, most hospitals in Spain observed similar waves [185], [271], [430], [431]. This evolution may reflect the impact of many factors, and an important one could be the control measures undertaken in our institution over the years [432].

The results of the multicentre study assisted by the Spanish Network for Infectious Diseases Research (REIPI), performed for 18 months (2008-2009) in 23 Spanish hospitals, revealed an average of methicillin resistance among *S.*

aureus of 25%. In 2006, a rate of 29.2% was reported in a point-prevalence study which was performed in 145 Spanish hospitals [271]. The European Antimicrobial Resistance Surveillance Network (EARS-Net) reported that the proportion of invasive isolates resistant to methicillin in 2009 was about 25.9% in Spain [433]. Other countries with a MRSA proportion above 25% were located in Southern Europe and on the British Isles [433]. In contrast, Nordic countries had low MRSA rates (<3%) [433]. The wide range of MRSA prevalence in Europe might be associated with the effectiveness of implementation of national antibiotic prescribing and infection control policies, among other factors [433], [434].

The latest data of the EARS-Net reported nine countries (Latvia, Luxembourg, Germany, United Kingdom, Belgium, France, Ireland, Hungary and Portugal) with decreasing trends for invasive MRSA, whereas an increasing trend was observed in four other countries (Sweden, Estonia, Austria and Romania) [185]. In any case, the rates above 22% detected in ten countries, Spain among them, are worrisome, with the MRSA problem being a public health priority [185].

Antimicrobial susceptibility of the MRSA clinical strains isolated in the HUB (1990-2014) and in other Spanish hospitals (2008-2009).

During the the first decade (1990-1999) of the study, the predominant pattern (82%) among MRSA strains isolated in the HUB involved resistance to erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampicin and tetracycline and was related to the Iberian clone [435]. This clone has been reported to be resistant to multiple antibiotics and susceptible only to

glycopeptides, cotrimoxazole, and the new antimicrobial agents such as linezolid and daptomycin [436]. In the following years, this multiresistant clone was gradually replaced by MRSA isolates that showed resistance only to tobramycin and ciprofloxacin combined with variable resistance to clindamycin and/or erythromycin. Since the late 1990s, similar resistance trends have been observed in other hospitals in Spain and in other countries such as France, Germany, Belgium or Portugal, with involvement of different clonal lineages. The Iberian clone practically disappeared in the 2005-2008 period [430], [431], [437]–[443], and we could not find any isolate related to this clone among the MRSA studied in the REIPI-multicenter study, performed in 2008-2009.

The evolution of antibiotic resistance was linked to the evolution of the different MRSA clones. For example, tetracycline and rifampicin resistance strains decrease significantly since 1995 along with the decrease of the Iberian clone. The emergence of a new MRSA phenotype in 2004 increased the rifampicin resistance rate from 21% (2000-2004) to 33% (2005-2009). This new phenotype of multiresistance differed from that of the Iberian clone on the low level of rifampicin resistance and on the tetracycline susceptibility, and was genetically related to the so called Southern Germany clone [425]. In 2014, only 2% of the MRSA isolates were rifampicin resistant, and again this was related with the disappearance of the Southern Germany clone.

In the REIPI-multicenter study conducted during 2008-2009, 31 different MRSA antibiotic resistance patterns were found, of which the most frequent were resistance to erythromycin, tobramycin plus ciprofloxacin, tobramycin plus ciprofloxacin or only ciprofloxacin in addition to beta-lactams. These resistance

patterns matched those observed in earlier studies performed in the HUB and in other Spanish hospitals [431], [444]–[446]. In this work 78% of MRSA isolates were non-multiresistant, as they were resistant to less than three antibiotics classes in addition to beta-lactams. This scenario is in agreement with the presence of the prevalent lineage CC5 harbouring SCC*mec* type IV, which carries no further resistance elements [112], [447].

A high rate of ciprofloxacin resistance (mean average: 96%) was a constant both, in the MRSA collection isolated from patients admitted to the HUB and in the REIPI-multicenter collection. High rates of ciprofloxacin resistance were detected in other Spanish studies and also in the last EARSS report [185], [445], [446]. Ciprofloxacin resistance is, in general, a marker of HA-MRSA among adult patients [431], [444].

Mupirocin resistance has been, in average, under 2% of the isolates obtained at the HUB (results not shown in chapter IV because it is not an antibiotic routinely tested against bacteremic isolates in our setting). Among isolates of the REIPI-multicenter study, mupirocin resistance accounted for 20% of all MRSA blood isolates and 35% of them were detected at a particular hospital in Madrid. Cuevas *et al*. communicated a similar mupirocin resistance percentage (18%) in a Spanish multicentre study, performed in 2002 [448]. However, in other countries mupirocin resistance remains under 9% [449]. Walker and colleagues, have shown rates of high-level mupirocin resistance following mupirocin usage, can disminished with restriction of mupirocin [450].

Comparative *in vitro* activity of vancomycin and daptomycin against MRSA blood strains isolated in the HUB and susceptibility of MRSA from other Spanish hospitals to these compounds.

S. aureus is the major cause of serious hospital- and community- acquired infections, and glycopeptides, particularly vancomycin (VA), have been the recommended therapy for serious MRSA infections for decades. MRSA spread has led to increased usage of glycopeptides and hence increased the selective pressure for the development of resistance. Consequently, reduced susceptibility to these compounds have been reported in *S. aureus* (both homogeneous and heterogeneous) [188], [193]. Daptomycin (DA) is an acidic lipopeptide that has demonstrated excellent activity against *S. aureus* and has activity against a small number of glycopeptide-intermediate *S. aureus* strains. Acquired resistance to DA in *S. aureus* is rare but it is being increasingly reported in relation to the use of this drug [214].

In the present study, we evaluated the activity of VA and DA (minimum inhibitory [MIC] and bactericidal [MBC] concentration) against MRSA blood strains of different lineages.

Among HUB MRSA isolates and during the first period (1990-1999), VA MIC_{90}/MBC_{90} values were significantly higher than in the second period (2000-2009), and this fact could be, at least in part, explained by the significant consumption of VA use for the treatment of severe MRSA infections from 1990 to 1999. A total of 23% of the strains tested exhibited tolerance to VA (MBC/MIC, ≥32). All isolates that showed an MIC of 2mg/L were VA-tolerant and all VA-tolerant isolates belonged to the Iberian clone (ST247-SCC*mec*I-

*agr*I). These findings are concordant with a previous study reported by our research group, that concluded that heteroresistant subpopulations of VA-susceptible *S. aureus* (hVISA) have been present in our hospital since 1990 and that heteroresistance may be a general and long standing property of the Iberian MRSA clone [189].

According to CLSI guidelines, susceptibility to VA is defined as an MIC ≤ 2mg/L [202]. Even with this definition of susceptibility, some studies reported the lack of relationship between MIC values, within the defined susceptible range, and VA clinical efficacy [451]–[453]. The vancomycin therapeutic guidelines, edited by the Infectious Diseases Society of America, recommended standard VA therapy only for patients whose MRSA isolates have an MIC<1mg/L [454].

Over our study we have observed that MICs of VA were not predictive of tolerance; however, the percentage of strains exhibiting tolerance increased as the MICs of VA increased. In this regard has been suggested that MBC instead of MIC could be of better use to predict VA tolerance, and could be useful for therapeutic decisions. However, MBC it is not routinely measured in clinical laboratories because it is labour intensive, doubles the turnaround time for results, and is not standardized [452], [455], [456].

Values of DA MIC_{90}/MBC_{90} among HUB isolates remained under 1mg/L over time. Tolerance to DA was not observed for any of the 185 isolates tested (in this selection all VA-tolerant strains were included). These results are consistent with previous studies pointing the daptomycin efficiency "*in vitro*" against MRSA and vancomycin-tolerant *S. aureus* strains [457], [458].

The REIPI-multicenter study allowed us to test susceptibility to VA and DA in parallel by two methods: E-test and broth microdilution (BMD). Regarding VA, the Etest provided vancomycin MIC results that were consistently higher (one or two dilution steps) than those provided by the reference BMD method. These findings are in line with those of previous studies [456], [459], [460]. In our opinion, as Rojas *et al*. stressed, there is lack of evidence in favor of using the E-test instead of the recommended microdilution method to assess MRSA vancomycin MIC [461]. Sadder and colleagues reported that daptomycin MICs were also affected by the method used but to a lesser degree (MICs were found to be 0.5 to 1 dilution step higher by the Etest method than by the reference method) and may also suffer from the perception of declining DA potency [462].

Acessory gene regulator function, *rpoB* mutations and vancomycin susceptibility in MRSA blood isolates.

A number of studies have linked reduction in *agr* function with vancomycin tolerance, reduction in the antibacterial activity, and the development of the hVISA or VISA phenotype. These changes being described in association with *agr* type II in the United States [463]–[467]. A clinical study also linked *agr* group II polymorphism with poor responses to vancomycin therapy for patients with MRSA infections [468]. In contrast, our results indicated that vancomycin tolerance was only related to the Iberian clone (ST247-SCC*mec*I) which presented *agr* type I, Apparently, *agr* type II isolates were dominant in the collection of strains used for describing *agr* dysfunction and reduction of VA susceptibility [465]. Other authors, described a collection of hVISA isolated from France and Belgium where the dominant *agr* group was type I [469].

In a number of *in vitro* studies, the reduction of the *agr* function has been shown to favour the development of vancomycin resistance and may confer a potential advantage in a hospital setting [465]–[467], [470]. Delta-hemolysin is a virulence factor regulated by *agr*: encoded by *hld* within the *agr* locus, it is a protein derived from translation of RNAIII, the effector molecule of *agr* [49]. In the present study, we found that 42% of *agr* type II and 35% of *agr* type I isolates presented a dysfunctional *agr* operon – negative delta-haemolysin synthesis, suggesting that *agr* dysfunction could be linked to the two *agr* groups and not with a single one. Interestingly, 85% of Southern-Germany (ST228-SCC*mec*I-*agr*II) MRSA isolates have demonstrated a loss of *agr* function (delta-haemolysin absence) in spite of their conserved susceptibility to VA.

Mutations of the *rpoB* gene are known mechanisms of rifampicin resistance in *S. aureus* and have been reported to contribute to the VISA phenotype [471]–[473]. We investigated the *rpoB* mutations in representative isolates of the two clones (ST247-SCC*mec*I-*agr*I and ST228-SCC*mec*I-*agr*II) showing resistance to rifampicin. The results of our study identified two *rpoB* mutations causing aa substitutions H481N and S529L in all studied isolates of Iberian clone, including VA-tolerant and no VA-tolerant isolates. On the other hand, rifampicin resistance was mainly due to *rpoB* mutation H481N in isolates of Southern Germany clone. Of note, this clone showed VA-MICs ≤ 1mg/L and did not show the VA tolerance phenotype. Our findings indicate that these *rpoB* mutations conferred resistance to rifampicin but were not responsible for vancomycin susceptibility. Screening for hVISA by the population analysis profile-area under the curve (PAP-AUC) method or the Etest macromethod should be performed to confirm our discordant findings [474].

Prevalence of *cfr*-mediated linezolid (LZD) resistance among MRSA in the HUB (1999-2010).

Since the introduction of LZD in the clinical practice several mechanisms of LZD-resistance have been described. The major mechanism of resistance is mediated by mutations in the V domain of the 23S rRNA [215], [216]. Another mechanism involved in LZD resistance is the RNA methylation by two different methyltranferases: RlnM - a codon insertion in the methyltransferase gene rlmN reduces LZD susceptibility in clinical *S.aureus*, and a methyltranferase encoded by the *cfr* gene [223], [224]. The *cfr* gene is mostly plasmid-located and confers cross resistance to phenicols, lincosamides, oxazolidinones, pleromutilines amd streptogramin A (PhLOPS phenotype). Some outbreaks of *cfr*-mediated LZD-resistant strains have been reported in the literature [219], [475]. LZD was introduced in the clinical practice in HUB during 2003. LZD prescription average in our hospital during the 2004-2010 period was of 0.28 defined daily dose (DDD) /100 patients-days, with a peak of 0.50 DDD/100 patients-days in 2009.

After screening for resistance to clindamycin and chloramphenicol, a potential *cfr*-mediated linezolid resistant phenotype, a single isolate of LZD MIC of 8 mg/L was positive for the *cfr* gene. The presence of the *cfr* gene has been described in different *S.aureus* genotypes either of community, ST8-MRSA-SCC*mec*IV and ST398-MRSA-SCC*mec*V, or nosocomial origin, ST125-MRSA-SCC*mec*IVc [220], [476], [477]. Thus, the presence of this mechanism of resistance in different *S.aureus* genotypes could be potentially spread worldwide due to its plasmid location.

In our series, overall LZD resistance mediated by the *cfr* gene is very low in this period, similar to other surveillance studies [478]. Among clindamycin and chloramphenicol resistant strains, *cfr*-mediated LZD resistance was 6.25% (1/16). Kerenberg *et al.* found the *cfr* gene in 3% of chloramphenicol-resistant strains of *Staphylococcus spp*. of animal and human origin [136].

The disc-diffusion technique is not suitable to recognize LZD resistance mediated by the *cfr* gene with the current CLSI breakpoints [202]. In our experience the detection of coincident resistance to clindamycin and chloramphenicol in a staphylococcal isolate would alert to the possibility of LZD resistance and could be used as a simple marker.

1.2. Molecular epidemiology description of emergent and endemic MRSA clones using genotypic and proteomic based methods.

Epidemiological characterization of MRSA isolates recovered in the HUB using different molecular and proteomic techniques, allowed us to detect important shifts in the clonal nature of endemic MRSA since 1990. In addition, the study of isolates from the REIPI-multicenter survey, carried out from 2008 to 2009, allowed to confirm that the evolution or MRSA clones has been similar in most of the hospitals included in the study.

Molecular characterization of the emergent and endemic MRSA clones by PFGE, MLST, SCC*mec* and *spa* typing.

During the 90's in Spain, ST247-SCC*mec*I (classically known as the Iberian clone) had been the most frequently observed type in the clinical environment. However, this clone has been gradually replaced by others, and currently, ST5-SCC*mec*IV and ST125-SCC*mec*IV are the predominant clones in Spanish hospitals [431], [441], [479]. The results of our study are in agreement with that trend and have revealed major shifts in the clonal nature of these isolates, namely, the massive replacement of the Iberian clone (ST247-SCC*mec*I, PFGE type A;B) by Paediatric related clones (ST5, ST125, ST146-SCC*mec*IV, PFGE type D;E) as the major lineage (41% of all 485 MRSA isolates studied) in HUB.

From 1990 to 1999 the majority of strains isolated in HUB, belonged to the multiresistant Iberian clone (ST247-SCC*mec*I-*agr*I) (see Figure 18). This clone, which is one of the most widely spread MRSA clones in Southern and Western Europe, displayed a SCC*mec* element that is commonly associated to HA-MRSA and have historically been associated with multiresistance (resistance to more than three antimicrobials) [431], [480]. The SCC*mec* type I found in representative isolates of the Iberian clone element remained highly conserved over time. This clone showed four different *spa* types out of which the *spa* t051 was the most frequent (see Figure 19). These results are in line with those of previous studies [264], [437], [481]. Since 1996, the Iberian clone was gradually replaced by isolates of CC5 (ST125, ST146; SCC*mec*-IV-*agr*II) related to the Paediatric clone (ST5-SCC*mec*IV-*agr*II). ST125 isolates have rarely been recovered worldwide, according to the *S. aureus* MLST database

(http://saureus.mlst.net). In fact, only 33 out of the 5322 isolates available from that database belong to ST125 (0.62% on 11/02/2015). Thirty of the isolates occurred in Spain and the other three in Norway, Finland, and France, respectively. The reason for the apparent evolutionary success clone ST125-SCC*mec*IV-*agr*II in Spanish hospitals is not clear, nor is it understood why that clone has not spread to other European countries. Regarding *spa* types distribution among ST5, ST125 and ST146 isolates, *spa* types t002 and t067 were dominant (77% of all ST5, ST125, ST146 strains group). Several studies reported that these two *spa* types were dominant in Spain [431], [446], [479], [482], [483]. The high frequency of t067 and t002 in Spain contrasted with the relatively low frequency (t067, 0.81%; t002, 6.93%) that were found in other countries (http://spa.ridom.de). Of note, the high frequency of t067 and t002 in Spanish hospitals limits the usefulness of *spa* typing for local investigations and makes it necessary to differentiate these frequent strains by other typing tools. The SCC*mec* type IV polymorphism was also conserved through the different clones included in these genotypes of CC5.

From 2004 to 2014, additional clones emerged in HUB: CC8 (ST8, ST450, ST2840-SCC*mec*IV-*agrI*) mostly associated with *spa* type t008; ST228-SCC*mec*I-*agr*II mainly associated with *spa* type t041 and ST22-SCC*mec*IV-*agr*I with t032 as the predominant *spa* type. These clones also showed a stable SCC*mec* structure over extended periods of time and have been described previously in studies carried out in our country and worldwide [431], [479], [482]–[486]. 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%) [484]. From 2004 to 2006 the Southern Germany (ST228-SCC*mec*I-*agr*II) clone was

associated to an MRSA outbreak in our setting [425]. This clone is was one of the major HA-MRSA clones widely disseminated around the world [237].

Notably, an important increase of CC8 (ST8, ST450, ST2840-SCC*mec*IV-*agr*I) isolates has been observed from 2003-2009 (26%, n=20/76) to 2010-2014 (74%, n=56/76) in HUB. Five isolates belonging to ST8-SCC*mec*IV-*agr*I were PVL positive and have been considered CA-MRSA. The substitution of HA-MRSA isolates by CA-MRSA isolates has already been reported by others [87], [239], [487], [488].

Finally, the most frequent SCC*mec* type found was SCC*mec* type IV, which was present in 65% of the isolates. Its presence in the predominant clones, in the sporadic clones, and in the community-acquired clones demonstrated successful persistence [260]. The successful introduction and persistence of the genetically shorter of SCC*mec*IV in the clinical environment has led to a global change in MRSA resistance profiles. Many studies have reported the gradual replacement of the classical multiresistant HA-MRSA clones by non-multiresistant isolates harbouring SCC*mec*IV [306], [437], [444], [447], [482], [489]. Furthermore, the analysis of *ccrB* typing was concordant with the results previously obtained by SCC*mec* typing. However, in order to predict SCC*mec* subtypes *ccrB* typing is not enough. The finding of *ccrB* allotype 412, previously described in coagulase-negative staphylococci (CoNS), suggests that CoNS may act as reservoir for the assembly and disseminations of the SCC*mec* elements [396].

The results of the molecular typing performed among the MRSA isolates (n=626) obtained at the REIPI-multicenter study were similar to those found

among HUB isolates in the same period (2008-2009). Overall, CC5 (ST5, ST125, ST146-SCC*mec*IV-*agr*II) was the most frequently detected clone (66%), followed by CC22 (ST22-SCC*mec*IV-*agr*I; 9%), CC8 (ST8-SCC*mec*IV-*agr*II; 7%) and CC5 (ST228-SCC*mec*IV-*agr*II; 6%).

The most frequent SCC*mec* type found was SCC*mec* type IV, which was present in 91% of the isolates. SCC*mec* type IV was identified in the predominant clones, but also in sporadic clones and in the community-acquired clones, suggesting a great degree of successful persistence [260]. SCC*mec* types I and II, which have historically been associated with multiresistance (resistance to more than three antimicrobials), were very uncommon in this study an in certain cases were associated with sporadic isolates.

SCC*mec* type V was only associated with the livestock-related MRSA clone, which was non typable by PFGE using the *Sma*I enzyme. It has been suggested that the use of zinc and others metals in animal feed formulations, can co-select SCC*mec* type V, carrying the *czrC* zinc resistance gene [337].

Concerning the clonal nature of our isolates, ST125-SCCmecIV clone emerged in Spain during 1996, although it was first reported in 2001, and has currently become predominant in Spanish hospitals [441], [479], [482], [484]. At a lower frequency, ST22-SCC*mec*IV, commonly named EMRSA-15 was the second most frequent clone detected in Spain during the studied period. This clone was first described in United Kingdom, where it became the major HA-MRSA clone, together with EMRSA-16, accounting for more than 95% of bacteraemia in this country [490]. It has also spread throughout Europe, being reported in Germany, Malta, Italy, and representing the major clone in hospitals

in Majorca, Czech Republic, and Portugal [264], [437], [491], [492]. Since 2010, this non-multiresistant clone ST22-SCC*mec*IV, and became the second major MRSA clone in our clinical setting. This clone is, as well, disseminated around the world [493], [494]. The third most frequent clone in our collection was the clone ST8-SCC*mec*IV-*agr*I and was mainly associated to non-multiresistant antibiotic profiles as previously reported in this clone [447].

Finally, 6% of the studied REIPI-multicenter isolates belonged to ST228-SCC*mec*I that is known as the Southern Germany clone. In contrast to ST8-SCC*mec*IV, this clone is associated to a multiresistant antibiotic pattern that differed from that of the Iberian clone on the low level RIF-R and on the tetracycline susceptibility. The Southern Germany clone had a large SCC*mec* type I that harboured several antibiotic resistance determinants. This clone was firstly reported in Germany in 1997-98 [495]. Since then, it has been reported in several European countries: Belgium, Slovenia or Switzerland [151].

Other minor community-acquired clones, accounting for 12% of all studied isolates, were identified. Twenty PVL positive isolates, 15 of them belonged to the ST8-SCCmecIV showing two distinct PFGE profiles (USA300, n=8; and USA300-ACME negative; n=7). These clones were the major source of community-acquired infections in the USA, Latin America, Canada, and Europe [111]. In Spain, USA300 strains are now resident in the community, but the reason for this specific epidemiology remains unclear, and it might be due to differences in predisposing conditions in the community and the high level of exchanges and migrants between Spain and Latin America [496]. The five remaining PVL positive isolates belonged to ST125 (n=2), ST8 (n=2) and ST30 (n=1) clones. A fatal case of necrotizing pneumonia in an an immunocompetent

adult patient, due to a CA-MRSA isolate of genotype ST30-SCCmecIV-*spa*t019 was described in Argentina [497]. This case highlighted the virulence traits of CA-MRSA clones and the importance of an accurate surveillance system of these genetic lineages. The livestock-associated clone (ST398-SCC*mec*IV/V-*agr*I) was another minor clone (n=10) identified in the REIPI-multicenter study.

An additional PHILOViZ clustering analysis of the distribution of each ST in Spain revealed that ST125, ST146 and ST8 were widely disseminated among the different autonomous communities of Spain. These results are in accord with previous studies indicating the spread of these clones across the Spanish hospitals [431], [441], [446], [482]. However, several STs such as ST5, ST45, ST435, ST30 and ST217 were only detected in Cataluña revealing the epidemic nature of these clonal lineages in Spain.

Regarding the *agr* types, our study revealed that most isolates presented *agr* type II (75%) were correlated to isolates that mostly clustered in the CC5 such as ST5, ST125, ST146-SCC*mec*IV and ST228-SCC*mec*I. *agr* types I and III were also found in our strain collection (23%) but in lower proportions than *agr* type II, and were correctly assigned to their clonal lineages. Monecke et al. previously reported this CC-*agr* type correlation [400].

It has been reported that MRSA spa-types mainly have a regional distribution in European countries [498]. In our work, the t067 *spa*-type was the most prevalent type among all 391 *spa*-typed strains and was the only type identified in all hospitals. These results are in line with those of previous studies, which reported that this *spa* type is the one most frequently found in Spain [479]. It has also been reported in Finland; however, it has been found at very

low frequency in other European countries, in the United States, and in South America [499], (http://spa.ridom.de/spa-t067.shtml). Another *spa*-type (t002) related to CC5 was identified in our collection. This *spa*-type has already been detected in other studies carried out in Spanish hospitals [431], [479], [482]. Additionally, this two *spa*-types was mainly associated with CC5 (ST5, ST125, ST146) and these results are in agreement with those obtained by Lozano *et al*. [446]. *spa* t008 was the third most frequently *spa* that was found in our isolates selection. This *spa* type have also been found in many Spanish hospitals and was associated with CC8 (ST8 and ST931). Several studies reported this *spa* type with the same CC association that we found in this work and is widely spread in worldwide [343], [500], [501], (http://spa.ridom.de/spatypes.shtml). Lastly, *spa* type t032 was only detected among isolates of CC22 (ST22 and ST217), the second major clone circulating in Spain during the studied period. Several reports revealed the spreading of this *spa* and the same clonal association [329], [485], (http://spa.ridom.de/spa-t032.shtml). Of note *spa* types t109 and t041 were only detected in the autonomous communities of Madrid and Cataluña respectively revealing the epidemic nature of these isolates. According to Ridom web page, t109 was mainly detected in Spain and in Germany, whereas t041 is widely spread in Europe and is related to the Southern Germany clone (ST228) (http://spa.ridom.de/spatypes.shtml).

Prevalence and molecular characterization of MRSA-ST398 isolated in the HUB.

Since 2005, several studies have been carried out focusing on livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) isolates, mainly on isolates of CC398 [242], [243], [331], [335], [336], [339], [341], [502]. Human colonization with LA-MRSA was first described among swine farmers in France and The Netherlands in 2003 [339], [346]. This is the first retrospective study of MRSA-ST398 in Spain covering a period from 2000 to 2011. In order to evaluate the prevalence and molecular characteristics of this clone in our hospital, one hundred and sixty-four isolates obtained between 2000 and 2011 from different sources were investigated. Prior to year 2000 Tet-R was very high among the MRSA isolated from the patients admitted to the HUB. This was due to the presence of an endemic clone – the Iberian clone (ST247) that was resistant to several antibiotics, including tetracycline. In the following years, tetracycline resistance dropped, often below 5%. Antibiotic susceptibility analysis revealed an unexpected diversity of resistance profiles among MRSA-ST398 isolates. In total, 14 different resistance patterns were seen with 10 of them represented by only one or two isolates. Of note, 49% of the isolates exhibited resistance to three or more antimicrobial agents, in addition to methicillin-resistance. Ciprofloxacin resistance was high (67%) considering the low percentage of resistance to this antibiotic exhibited by other MRSA community clones. Spanish and Belgian studies described resistance to fluoroquinolones of 58% and 83% respectively [343], [502]. However, in other European, USA or Canadian studies, the resistance percentage to fluoroquinolones is very low or even inexistent [241], [331], [333]. The

veterinary use of quinolones in Spain is difficult to assess. The public data available on the website of the Spanish Ministry of Health (*Ministerio de Sanidad, Politica Social y Igualdad*) (www.aemps.gob.es) only describes the total amount of antimicrobials sold for veterinary purposes, and does not distinguish between the therapeutic use of antibiotics and the use of antibiotics to enhance growth. In any case, total sales of quinolones was about 50 tons in 2009, much lower than the sales of other antibiotics such as tetracyclines (350 tons), sulfonamides (250 tons) or beta-lactams (180 tons) during the same year.

The combination of *tet*(K) and *tet*(M) resistance genes was commonly seen among the 33 MRSA-ST398 isolates. The methylase *erm*(C) gene was present in 10 out of 11 erythromycin resistant strains. Resistance to cotrimoxazole was found in a single isolate, whereas in other MRSA-ST398 studies cotrimoxazole resistance was more prevalent and in some cases exceeded 80% [502], [503].

Some strains showed the unusual erythromycin-susceptibility/clindamycin-resistance pattern. This phenotype seems to be related to animal clonal lineages of *S. aureus* animals and has been associated with the presence of plasmid-borne resistance genes *vga*(A), *vga*(C), *lnu*(A) or *lnu*(B) [504], [505]. Some resistance genes described in MRSA CC398 strains such as *erm*(T), *vga*(B), *vga*(C), *lnu*(B), *dfrSI, dfrK, dfrG* and *tet*(L) were not included in the DNA microarray applied in this study [503].

The dominant *spa* type t011 in our collection has been commonly found in other studies, both in Spain, and in other countries [80], [243], [244], [330], [343]. There was a single isolate of *spa* type t899, with only two repeats in

common with t011. Therefore, this t899 isolate did not cluster in *Spa*-CC011 after BURP analysis. As described previously, this could be explained by the acquisition of a large DNA region which includes the *spa* gene, from a remote *S. aureus* clone. *spa* type t899 has been described in association to ST9, a clone also described in samples of animal origin [80], [506]. The linkage of the same *spa* type in two different ST types would suggest exchange of genetic material between two clones of animal origin.

The presence of genes coding for virulence factors was very poor in our collection. None of the studied isolates carried the PVL - encoding genes *lukF-PV* and *lukS-PV*, contrasting to studies from Sweden and China that report PVL-positive isolates in patients who had no previous contact with animals [332], [507]. Generally, MRSA-ST398 lacks certain important virulence factors for humans [336]. In our study genes involved in immune evasion (*scn*, *sak a*nd *chp*) were only detected in the single t899 isolate. These virulence factors are active only against the innate immune system in humans [68]. The lack of these virulence factors may partially explain why these strains do not appear to be highly infectious for humans, and usually are associated with SST infections [243]. However, a few severe infections by ST398 have been sporadically published in several countries, such as pneumonia or bacteraemia [242], [244]. Even though the majority of the patients in our study were colonized by MRSA-ST398 (70%), four SST infections and four respiratory tract infections were detected in our series. In addition, two invasive infections, bacteraemia and subdural empyema, were also detected, in patients showing previous nasal colonization. Death was related to the MRSA-ST398 infection in the bacteremic patient: an 84-year-old woman who was hospitalized in the HUB because of a

thoracic aortic aneurysm. One month after the surgery, the patient experienced a febrile episode and MRSA-ST398 was recovered from blood and from the central venous catheter tip. The isolate was resistant to tetracycline and beta-lactams. One week after treatment with vancomycin, the patient died.

The isolates causing infection did not differ from the colonizing isolates, regarding genotype, virulence or antibiotic resistance profile.

In our study, some data could not be collected properly such as the contact with animals, a risk factor for infection by LA-MRSA. However, a high percentage (73%) of the patients lived in or near to a rural environment. Another limitation was the selection of the isolates to be studied by the presence of tetracycline resistance. Although this is a common feature among MRSA-ST398, this approach could underestimate the number of isolates belonging to CC398 in HUB [343].

In conclusion, in the last two years (2010-11) the number of MRSA-ST398 isolates infecting or colonizing patients increased significantly in our setting, as well as the increase in tetracycline resistance. The emergence of this clonal lineage has also been reported in other countries in Europe [242], [243], [330], [508]. These studies showed a remarkable increase in the proportion of LA-MRSA isolates, including outpatients and primary health care patients, which were not covered in our hospital-based study. According to our results, the majority of studied isolates carried the genes encoding haemolysins and adhesion cellular factors, but other virulence factors usually found among *S. aureus* were not detected. Phenotypic expression of antibiotic resistance was variable among the MRSA-ST398 isolates and nearly half of the isolates were

resistant to multiple antibiotics. In addition, patients harbouring this clone were often debilitated by underlying diseases, such as cancer. Due to the increased public health interest about MRSA-ST398, further studies should be conducted to record risk factors from infected or colonized patients by this lineage such as routes of transmission and association with animals.

The increased rate of MRSA-ST398 clone observed from 2010 to 2011 in our setting, lead us to the prospective surveillance of this microorganism during 2012 and 2013. Recently, a multicentre study carried out in Spain, described the increased rate of MRSA-ST398 among CA-MRSA during 2012 [325]. We described a similar trend in our clinical setting, where MRSA-ST398, in spite of low numbers, represented 48% of tetracycline resistant MRSA strains isolated between 2010 and 2011 [509]. However, we find a similar proportion of MRSA-ST398, 37%, during the 2012-2013 period, representing 1.4% of all MRSA. Higher proportions of MRSA-ST398 among MRSA clinical isolates (5%) were described in a different Spanish region, as well as in other European countries that also carried out their studies in areas with high levels of livestock production, i.e. Netherlands 11.9% or Belgium 4.7% [330], [343].

Most of the MRSA-ST398 isolates were *spa* type t011 which is the predominant *spa* type among pig-associated isolates often reported in Spain and other countries [80], [243], [244], [330], [343], [509]. A single isolate was ascribed to *spa* type t899 and did not cluster in *spa*-CC011 by BURP analysis. Price *et al*. reported that the strains carrying *spa* type t899 did not follow the whole genome sequencing phylogeny, and this fact could be explained by the acquisition of a large DNA region including SCC*mec* and the *spa* gene from a

ST9 donor [80]. The linkage of the same *spa* type in two different ST types would suggest exchange of genetic material between two clones of animal origin. All isolates but one carried the SCC*mec* type V. It has been suggested that the use of zinc and others metals in animal feed formulations, can co-select SCC*mec* type V, carrying the *czrC* zinc resistance gene [337].

Antibiotic susceptibility analysis provided evidence of a variety of resistance profiles among MRSA-ST398 isolates. The diversity of antimicrobial resistance phenotypes and genotypes among MRSA-CC398 strains has been reported worldwide and results from the capability of this lineage to acquire external DNA combined with the antimicrobial selection pressure applied to the microorganisms in diverse environments [336], [504]. Of note, ciprofloxacin resistance was detected in 13 out of 16 isolates (81%), in contrast to other MRSA community clones which exhibit a low resistance rate to this antibiotic [325]. All MRSA-CC398 were Tet-R, this fact do reflect the antimicrobial veterinary use in our country. The European Medicines Agency reported Spain to be the second country consuming veterinary antimicrobial agents in 2012, with sales of tetracyclines, penicillins, macrolides, lincosamides, and polymyxins accounting for 82.7% and quinolones accounting for 3.4% of the various veterinary antimicrobial classes sold in Spain during 2012 [510].

Resistance to macrolides-lincosamides was mainly mediated by *erm*(C) gene. An unusual erythromycin-susceptibility/clindamycin-resistance phenotype was detected in a single isolate. Our strain lacked *vga*(A) and *lnu*(A) genes. However, other genes such as *vga*(C) and *lnu*(B) that could be associated to this phenotype were not tested in our study [504], [505].

In the context of the CC398, the absence of *tet*(M) has been considered a marker of human origin [80]. In our study, the single isolate (patient number 12) carrying only *tet*(K) and lacking *tet*(M), could thus be derived from a human-associated lineage [80], [511]. Interestingly, only two isolates (patient numbers 7 and 12) presented the genes encoding the IEC protein complexes (*scn*, *chp* and *sak*) located on φ3 bacteriophages that are exclusively carried in human CC398 isolates [512], [513]. In contrast to a previous study, the IEC genes were not only detected in an invasive isolate but also in a human colonization isolate, suggesting that these genes they don´t play an essential role in invasive disease [513]. Molecular findings could suggest that these two isolates belonged to two different clades: one carrying both ICE and *tet*(M) genes (patient number 7) and other (patient number 12) only carrying the ICE genes [511]. However, further in-depth analysis is needed to determine whether the two aberrant strains belong to the human- or livestock-adapted CC398 lineage. Moreover, the low frequency of these IEC genes indicates that they are not crucial for human adaptation of CC398 and could help to understand why these strains are less virulent in humans than other clone lineages and are most commonly associated with SST infections [80], [243], [513]. In general, this MRSA-ST398 clone typically lacks important virulence genes, including the PVL gene, which is in accordance with our results [245], [336]. Only a few studies reported PVL-positive strains isolated from patients with or without previous contact with livestock [332], [507], [514]. Moreover, the majority of the studied MRSA-ST398 isolates shared the same genes for biofilm production and genes encoding MSCRAMMs and were similar to those described from other CC398 strains [515], [516].

The phenotype and genotype of isolates causing infection in our study did not significantly diverge from the colonizing isolates. The patients carrying MRSA-ST398 in HUB were admitted to the hospital to undergo surgical procedures, to be treated for an underlying disease (mainly cancer or cardiovascular dysfunction) or both. In four infected patients, colonization by the MRSA-ST398 was detected at admission prior to the development of the infection. Two more patients acquired the infection while admitted to the hospital. However, no other nosocomial transmitted cases were generated, and no carriers were found among hospital staff. Also of note, is the persistent carriage found in some patients and in one case the nasal colonization was invariably detected for more than five years.

Limitation to our study was the difficulty in assessing the patients exposure to animals, a risk factor for carriage of MRSA-ST398 [341]. However, colonization or infection by MRSA-ST398 have been reported in persons lacking identified livestock-associated risk factors [245], [333].

In conclusion, our study shows that the MRSA-ST398 clone was present in the Southern Barcelona Metropolitan area, although in low numbers, and patients attending the hospital showed colonization mainly of community origin. In three cases (two infections and one tracheostomy colonization), hospital transmission was hypothesized since the patients were repeatedly negative for nasal carriage, prior to the isolation of the MRSA-ST398 from a clinical sample. Interestingly, genetic analysis revealed two MRSA-ST398 isolates with molecular patterns of human re-adaptation.

Automated categorization of MRSA clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry.

Previous studies introduced the use of MALDI-TOF/MS to discriminate MRSA clones or clonal lineages using different MALDI-TOF/MS approaches [355], [356], [418]. Wolters *et al*. described a preliminary MALDI-TOF-based MRSA typing scheme including 13 specifics peaks that allowed the discrimination of 5 MRSA CCs (CC5, CC8, CC22, CC30 and CC45) [355]. However, this strategy had several limitations that made it difficult to implement in routine analysis, such as: no clear criteria for peak selection, tedious binary conversion and the requirement to generate hierarchical clusters for CC designation. More recently, Josten *et al.* evaluated the use of MALDI-TOF/MS to differentiate 6 *S. aureus* CCs (CC5, CC22, CC8, CC45, CC30 and CC1) by correlating peak shifts to point mutations, i.e., the genotypes of the strains [356]. Yet again, the methodology used required the close examination of spectra by expert personnel, thus making its implementation into routine diagnostic analysis not feasible.

In this study, we have evaluated for the first time a strategy that relies on a robust statistical analysis and the automated use of MALDI-TOF/MS to discriminate the major MRSA clonal lineages identified in our hospital. The ClinProTools statistical software was applied to generate and validate a pattern recognition SNN model that allowed the classification of MRSA epidemic clones predominant in our area with a sensitivity and specificity of 100% and 99.11%, respectively, providing a PPV of 99.6%. The SNN model identified eleven peaks with the highest separation power between all four CCs (3,278, 3,445, 3,876,

3,891, 4,514, 4,540, 4,939, 5,004, 6,481, 6,553, and 6,591 *m/z*). Some of such peaks matched with those previously identified by Wolters *et al*. (3,276, 3,876, 4,511, 5,002 and 6,591 *m/z*) and Josten *et al*. (3,875, 3,891, 4,511, 5,002, 6,552, and 6,592 *m/z*), thus reinforcing the discriminatory value of our analysis [355], [356]. Interestingly, those peaks showing a greater contribution to the model in our study (3,876, 3,891, 4,514, 5,004, 6,553 and 6,592 *m/z*) were already highlighted by Josten *et al.,* and peaks 3,891, 5,004 and 6,592 have been identified as respective biomarkers for CC5, CC22 and CC8 in both studies. Of note, peak 4,511 was identified as a biomarker for CC398 in our study while Josten *et al.* identified this peak as a biomarker also for CC30, CC45 and CC88, which were not included in our model due to low number of strains in our collection to achieve statistical significance. Close inspection of spectra from representative isolates belonging to such CCs identified the presence of peak 4,511, confirming the results by Josten *et al* [356].

Nevertheless, while our SNN model shows excellent specificity and sensitivity, it has the same flaws of precedent studies. It requires tedious and expert analysis and it is not suitable for routine analysis. In addition, the ClinProTools software is not freely available and may be out of reach to some laboratories. The SNN model, however, allowed us: i) to identify the most relevant peaks that discriminate between the main 4 CCs present in our local area, ii) use this information to adjust the weight of those peaks to generate specific subtyping MSPs with the MALDI Biotyper software and, iii) allocate test strains into their proper CCs, basically using the same procedure that is already implemented in many centres for species identification, without any further analysis.

The automated approach proved to be highly successful in the correct identification of isolates belonging to CCs included in the SNN model (PPV of 98.9%) and although it misidentified isolates from other CCs, it usually provided logscore values below our arbitrary threshold. Nevertheless, a few isolates belonging to CC30 (n=3), CC45 (n=1) and CC80 (n=1) were incorrectly allocated to CC398 with logscore values above the threshold, likely due to the presence of the 4,511 *m/z* peak that was identified in our model as a specific biomarker for CC398 but that is present in those CCs as well. We are aware that a recalculation of the model including representative strains from other CCs is needed to overcome this limitation. There were no relevant differences between the spectra from invasive vs. non-invasive isolates of CC398, although the sample size was too small to perform a robust analysis. Differences between all isolates could only be attributed to their categorisation into different CCs.

In summary, we have established a simple, automated and accurate methodology for the classification of MRSA clinical isolates into different CCs by MALDI-TOF/MS, which removes the need for batch or retrospective epidemiological analysis and allows interlaboratory comparison. While further studies are needed to include additional CCs, we find this novel automated MALDI-TOF/MS approach to be a promising, powerful and reliable tool for *S. aureus* typing that could easily be implemented in routine laboratories already using mass spectrometry.

1.3. Antibiotic resistance and virulence genetic determinants of MRSA bacteremic clones.

S. aureus is a versatile pathogen capable of causing a wide range of human diseases. The intrinsic arsenal of virulence determinants and the ability of acquiring antibiotic resistance in the face of antimicrobial challenge have enabled it to remain an ongoing, significant human pathogen.

Virulence and resistance determinants characterization of endemic MRSA strains analysed by DNA-Microarray Genotyping.

The DNA microarray genotyping (DMG) methodology [304], [354], [400] allowed us to study a large number of resistance genes and pathogenicity markers in a large number of *S. aureus* strains.

In this study, we investigated the virulence and the antibiotic resistance determinants of 130 MRSA blood isolates, representing the major MRSA clones circulating in the HUB.

All MRSA isolates were correctly assigned to their respective CC and *agr* polymorphism. However, SCC*mec* was not solved in 13% of the studied isolates. A possible explanation for this might be that these clones have an atypical resistance and virulence determinants profile, thus the array could not correctly identify the isolates. Another drawback was the assignation of ST247 to strains belonging ST1819. ST1819 is a single locus variant of ST247. Therefore, this technique is not reliable for identifying new genotypes or some epidemic clones. Kuhn *et al.* discussed these limitations previously [517].

Overall, antimicrobial resistance genes detected by array were according to phenotypic expression. As we expected, all isolates presented the *mec*A gene and *bla*Z gene, which encodes the PBP2A and beta-lactamase gene respectively. Macrolide resistance genes were variable according to their clonal nature and resistance pattern. For instance, ST247/ST1819, ST8 and ST228 with MLS_B phenotype harboured the *ermA* gene while ST125, ST146 and ST22 harboured the *ermC* gene. *msrA* gene was associated to the MS_B phenotype, and was mainly detected in strains belonged of ST125 and ST146. These genes are commonly associated to macrolides resistance among MRSA strains [518]. It has been suggested that macrolide/lincosamide resistance genes could have a geographical distribution, however was not possible to perform this analysis in this study [336].

Aminoglycoside resistance (gentamicin and tobramycin) is mediated by the bifunctional modifying enzyme *aacA-aphD*, usually accompanied by the *aadD* gene. These resistance determinants were present in ST247, ST1819, ST228 and in a few isolates of ST125 and ST146. Of note, most of the S125 and ST146 isolates that showed tobramycin resistance were positive for the *aadD* gene. The presence of an aminoglycoside resistance gene(s) detected by array profiling could be used to accurately predict an isolate's phenotypic aminoglycoside resistance pattern. However, the phenotypic expression of aminoglycoside resistance could not always be used to infer which aminoglycoside resistance gene(s) was present [519].

Tetracycline resistance was commonly based on the presence of *tetM* in all isolates of ST247/ST1819. *tetK* gene was rarely detected in our isolates. *tetM* genes confer resistance to tetracycline and minocycline and were the most

prevalent resistance determinants in MRSA The association of resistance genes with mobile genetic elements possibly enhances the spread of resistant traits in MRSA [520].

The gene *dfrA*, related to co-trimoxazole resistance, was found in isolates of the Iberian clone both, resistant and susceptible to the antibiotic. Apparently, although the gene could be detected by the array it did not have effect on phenotypic expression of co-trimoxazol resistance. Gene *dfrA* was raraly described in MRSA [521]. Likewise, chromosomal *fosB*, detected in all CCs except in CC22, was not associated withfosfomycin resistance. In this case, the probe included in the microarray was an incomplete gene fragment intended to differenciate hybridization profiles. Other authors, reported similar results [522].

Regarding virulence genes, *tsst-1* gene was detected in a single isolate of ST125. Interestingly, this toxin is mostly found in ST30-MSSA isolates. However, several studies performed in different countries have been reported this toxin in other MRSA genetic backgrounds such as CC30, C22 and CC80 [523]–[525].

ST228 was the only clone that was positive for exfoliative toxins *etA, etB*. These toxins are responsible for staphylococcal scalded skin syndrome (SSSS) [526]. The prevalence of these genes might be due to the specific geographic region [527].

ST247 and ST125 could be distinguished by their enterotoxins profiles. ST247 carried *ent*A and ST125 presented more enterotoxins genes such as *ent*G, I, M, N, O, and U genes. These gene factors may conferred s selective

advantage and could explain why ST125 is a more successful clone than the Iberian clone [528].

In addition *S. aureus* also produces a family of exotoxins named superantigen like proteins (*ssl* genes) that seemed to be involved in virulence [400], [529]. These genes are present in all MRSA studied, but individual genes are variable, resulting a strain-specific pattern, Monecke et al, described the usefulness of this genes to distinguish CC5 from CC8 [400]. In our study, the presence or absence of these genes also allowed to discriminate among CC5, CC8 and CC22.

All isolates showed the same capsule (capsule type 5) and biofilm genes profile. It means that all isolates were positive for adherence (*icaA*, *icaC* and *icaD* genes) but were negative for the *bap* gene encoding a surface protein involved in biofilm formation. The "*microbial surface components recognizing adhesive matrix molecules*" (MSCRAMM) profile and *cna* genes, were enough to identify CC22, based on the absence of some MSCRAMM genes (*ebh*, *fib*, *fnbB*) and the unique presence of *cna* gene. These findings are in line with those of previous studies [530]. Finally, some isolates of CC5 showed deletions of MSCRAMM genes. Monecke et al described that partly deletions of MSCRAMM genes could be observed in CC5 group [95]. Although virulence gene expression may also affect the clinical outcome, it is difficult to predict the impact of genes expression *in vivo* [530].

Chapter VI

CONCLUSIONS

Conclusions

1. The global average of methicillin resistance among *S. aureus* in Hospital Universitari de Bellvitge (HUB) from 1990 to 2014 was 20%. Over the last 10 years, methicillin resistance percentage was around 24%. This figure is similar in other hospitals in Spain.

2. From 1990 to 1999 the dominant lineage in HUB was the multiresistant Iberian clone (ST247-SCC*mec*I-*agrI*) and its genetic background remained highly conserved over extended periods.

3. The Iberian clone was gradually replaced by isolates of Clonal Complex (CC) 5 (ST125 and variants; SCC*mec*-IV-*agr*II) related to the Pediatric clone (ST5-SCC*mec*IV-*agrII*). CC5 has been the dominant lineage from 2000 to 2014.

4. Over the last 10 years additional endemic clones emerged, such are ST8-SCC*mec*IV-*agr*I, ST22-SCC*mec*IV-*agr*I and ST228-SCC*mec*I-*agr*II. Of these, ST8-SCC*mec*IV-*agr*I has become the second most frequent lineage in 2014.

5. The major antibiotic resistance profiles of contemporary isolates in the HUB included resistance to tobramycin and ciprofloxacin combined with resistance to erythromycin and/or clindamycin. These resistance phenotypes were commonly shared by isolates of the dominant genetic lineages, CC5 and ST8.

6. The presence of specific antibiotic resistance determinants was related to particular clones. Macrolide resistance of ST247, ST1819, ST8 and ST228 strains with a MLS_B phenotype was due to the presence of *erm*A gene; while *erm*C gene was the responsible for the MLS_B phenotype in ST125, ST146 and ST22 strains.

The MS_B phenotype, which is mediated by *msrA* gene, was observed in strains of ST125, ST146 and in some ST8 strains.

Aminoglycoside resistance (gentamicin and tobramycin) was mainly codified by the bifunctional modifying enzyme *aac*A_*aph*D, generally accompanied by the *aadD* gene in isolates of ST247, ST1819 and ST228; while tobramycin resistance encoded by *aadD* gene was found in most isolates of ST125 and ST146.

7. The genetic polymorphisms of the SCC*mec* element have remained stable over the study period within each genetic lineage.

8. The majority of the MRSA positive for the Panton-Valentine leukocidin isolated in the HUB, belonged to clone the ST8-SCC*mec*IV-*agr*I.

9. The first isolate belonging to the MRSA-ST398 clone (ST398-SCC*mec*V-*agr*I) was identified in the HUB in 2003. Isolates of MRSA-ST398 showed a low virulence gene content, were always resistant to tetracycline and had variable resistance profiles to other antibiotics. Patients colonized or infected by MRSA-ST398 were often debilitated by underlying comorbidities.

10. Discrimination of MRSA isolates into major Clonal Complexes was possible using the MALDI-TOF/MS system. Automated resolution of patterns eliminated the need for working on batches or retrospective epidemiological analysis and allowed interlaboratory comparison.

11. The *in vitro* phenomenon of vancomycin (VA) tolerance was only found among isolates of Iberian clone (ST247-SCC*mec*I-*agrI*). On these isolates, studies of VA-MBC (minimum bactericidal concentration) were more accurate

on predicting *in vitro* activity of VA against MRSA than VA-MIC (minimum inhibitory concentration) itself.

12. Reduced susceptibility to VA was a lineage-related property in the collection of isolates studied in this work. There was no detected relationship with the *agr* dysfunction, *agr* type or *rpo*B mutations.

13. Daptomycin showed an excellent *in vitro* bactericidal activity against different MRSA isolates in spite of their clonal nature.

14. Linezolid resistance was very low in our study and *cfr*-mediated resistance was found in a single case. Staphylococcal isolates exhibiting a phenotype of resistance to clindamycin, chloramphenicol and linezolid could be suggestive of *cfr*-mediated linezolid resistance.

15. Combination of virulence factors was associated with each clone. Isolates of CC5 and CC22 presented more virulence determinants than isolates of CC8. This fact might help enhancing the invasive capacities of these CC.

16. Molecular epidemiology and antibiotic resistance profiles of MRSA isolates in other hospitals in Spain, were similar to those found in the HUB in the same frame of time (2008-2009):

i) The average of methicillin resistance among *S. aureus* from other Spanish hospitals in 2009 was 25%.

ii) More than half of the studied isolates from other hospitals belonged to the CC5, mainly to ST125-SCC*mec*IV-*agr*II.

iii) The most common phenotypes of antibiotic resistance included resistance to tobramycin, ciprofloxacin and/or erythromycin.

iv) Clones of community origin accounted for less than 5% of the isolates, and belong mainly to the USA300 clone (ACME positive and negative) and MRSA-ST398 clone.

Chapter VII

REFERENCES

References

[1] **Que, Y. A., and P. Moreillon.** 2010. *Staphylococcus aureus* (including Staphylococcal Toxic Shock), p. 2543–2578. *In* G. L. Mandell, J. E. Bennett and R. Dolin (eds.), Mandell, Douglas and Bennett's principles and practice of infectious diseases, 7th ed. Churchill Livingstone, Philadelphia, USA.

[2] **Ogston, A.** 1882. Micrococcus Poisoning. J Anat Physiol 17:24–58.

[3] **Ryan, K. J.** 2004. Staphylococci, p. 261–272. *In* K. J. Ryan and C. G. Ray (eds.), Sherris medical microbiology: an introduction to infectious diseases, 4th ed. McGraw-Hill, USA.

[4] **Cueto, M., and A. Pascual.** 2009. Microbiología y patogenia de las infecciones producidas por *Staphylococcus aureus*, p.16-17. *In* A. Pahissa (ed.), Infecciones producidas por *Staphylococcus aureus*, 1st ed. ICG Marge, SL., Barcelona, Spain.

[5] **Euzéby, J. P.** 1 August 2015, posting date. List of Prokaryotic names with Standing in Nomenclature. http://www.bacterio.cict.fr/s/staphylococcus.html. [Online].

[6] **Bannerman, T. L., and S. J. Peacock.** 2007. *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci, p. 390-411. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller, (eds.), Manual of clinical microbiology, 9th ed. ASM Press, Washington DC, USA.

[7] **Gotz, F., T. L. Bannerman, and K. Schleifer.** 2006. The genera *Staphylococcus* and *Macrococcus*, p.1-75. *In* M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer, and E. Stackebrandt (eds.), The Prokaryotes, 3rd ed. Springer, New York, USA.

[8] **Kwok, A. Y., and A. W. Chow**. 2003. Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial hsp60 gene

sequences. Int J Syst Evol Microbiol **53:**87–92.

[9] **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev **36:**407-77.

[10] **Novick, R.** 1993. *Staphylococcus*, p. 17-33. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (eds.), *Bacillus subtilis* and other Gram-positive bacteria: biochemestry, physiology, and molecular genetics, 1st ed. ASM Press, Washington DC, USA.

[11] **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. I. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu.** 2002. Genome and virulence determinants of high virulence community-acquired MRSA. Lancet **359:**1819–27.

[12] **Lindsay, J. A., and M. T. Holden.** 2006. Understanding the rise of the superbug: Investigation of the evolution and genomic variation of *Staphylococcus aureus.* Funct Integr Genomics **6:**186–201.

[13] **Lindsay, J. A., and M. T. Holden.** 2004. *Staphylococcus aureus*: superbug, super genome?. Trends Microbiol **12:**378–85.

[14] **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day.** "How clonal is *Staphylococcus aureus*?. J Bacteriol **185:**3307-16.

[15] **Thomas, C. M., and K. M. Nielsen.** 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol **3:**711–21.

[16] **Lindsay, J. A.** 2010. Genomic variation and evolution of *Staphylococcus aureus.* Int J Med Microbiol **300:**98–103.

[17] **Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh, and J. L. Nouwen.** 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis **5:**751–62.

[18] **Esposito, S., L. Terranova, A. Zampiero, V. Lerardi, W. P. Rios, C. Pelucchi, and N. Principi.** 2014. Oropharyngeal and nasal *Staphylococcus aureus* carriage by healthy children. BMC Infect Dis **14:**723.

[19] **Marshall, C., and D. Spelman.** 2007. Is throat screening necessary to detect methicillin-resistant *Staphylococcus aureus* colonization in patients upon admission to an intensive care unit? J Clin Microbiol **45:**3855.

[20] **Nilsson, P., and T. Ripa.** 2006. *Staphylococcus aureus* throat colonization is more frequent than colonization in the anterior nares. J Clin Microbiol **44:**3334–39.

[21] **Kluytmans, J.** 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **10:**505–20.

[22] **Valentine, F. C., and S. P. Hall-Smith.** 1952. Superficial staphylococcal infection. Lancet **2:**351–4.

[23] **von Eiff, C., K. Becker, K. Machka, H. Stammer, and G. Peters.** 2001. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. Study Group. N Engl J Med **344:**11–6.

[24] **Nouwen, J., H. Boelens, A. van Belkum, and H. Verbrugh.** 2004. Human factor in *Staphylococcus aureus* nasal carriage," Infect Immun **72:**5442–48.

[25] **Albrich, W. C., and S. Harbarth.** 2008. Health-care workers: source, vector, or victim of MRSA? Lancet Infect Dis **8:**289–301.

[26] **Kooistra-Smid, M., M. Nieuwenhuis, A. van Belkum, and H. Verbrugh.** 2009. The role of nasal carriage in *Staphylococcus aureus* burn wound colonization. FEMS Immunol Med Microbiol **57:**1–13.

[27] **van Belkum, A., N. J. Verkaik, C. P. de Vogel, H. A. Boelens, J. Verveer, J. L. Nouwen, H. A. Verbrugh, and H. F. Wertheim.** 2009. Reclassification of *Staphylococcus aureus* nasal carriage types. J Infect

Dis **199:**1820–26.

[28] **Nouwen, J. L., M. W. Fieren, S. Snijders, H. A. Verbrugh, and A. van Belkum.** 2005. Persistent (not intermittent) nasal carriage of *Staphylococcus aureus* is the determinant of CPD-related infections. Kidney Int. **67:**1084-92.

[29] **Nouwen, J. L., A. Ott, M. F. Kluytmans-Vandenbergh, H. A. Boelens, A. Hofman, A. van Belkum, and H. A. Verbrugh.** 2004. Predicting the *Staphylococcus aureus* nasal carrier state: derivation and validation of a "culture rule". Clin Infect Dis **39:**806–11.

[30] **Eriksen, N. H., F. Espersen, V. T. Rosdahl, and K. Jensen.** 1995. Carriage of *Staphylococcus aureus* among 104 healthy persons during a 19-month period. Epidemiol Infect **115:**51–60.

[31] **Hu, L., A. Umeda, S. Kondo, and K. Amako.** 1995. Typing of *Staphylococcus aureus* colonising human nasal carriers by pulsed-field gel electrophoresis. J Med Microbiol **42:**127–32.

[32] **Vandenbergh, M. F., E. P. Yzerman, A. van Belkum, H. A. Boelens, M. Sijmons, and H. A. Verbrugh.** 1999. Follow-Up of *Staphylococcus aureus* nasal carriage after 8 years : redefining the persistent carrier state. J Clin Microbiol **37:**3133–40.

[33] **den Heijer, C. D., E. M. van Bijnen, W. J. Paget, M. Pringle, H. Goossens, C. A. Bruggeman, F. G. Schellevis, and E. E. Stobberingh.** 2013. Prevalence and resistance of commensal *Staphylococcus aureus*, including meticillin-resistant *S. aureus*, in nine European countries: A cross-sectional study. Lancet Infect Dis **13:**409–15.

[34] **Peacock, S. J., I. de Silva, and F. D. Lowy.** 2001. What determines nasal carriage of Staphylococcus aureus? Trends Microbiol **9:**605–10.

[35] **Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond, and A. Peschel.** 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. Nat Med **10:**

243–5.

[36] **Wertheim, H. F., E. Walsh, R. Choudhurry, D. C. Melles, H. A. Boelens, H. Miajlovic, H. A. Verbrugh, T. Foster, and A. van Belkum.** 2008. Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. PLoS Med **5:**0104–12.

[37] **Loeffler, A., D. U. Pfeiffer, D. H. Lloyd, H. Smith, R. Soares-Magalhaes, and J. A. Lindsay.** 2010. Meticillin-resistant *Staphylococcus aureus* carriage in UK veterinary staff and owners of infected pets: new risk groups. J Hosp Infect **74:**282-8.

[38] **Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin, and R. P. Novick.** 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology **154:**2265–74.

[39] **Liu, G. Y.** 2009. Molecular pathogenesis of *Staphylococcus aureus* infection. Pediatr Res **65:**71–7.

[40] **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N Engl J Med. **339:**520–32.

[41] **Archer, G. L.** 1998. *Staphylococcus aureus* : a well-armed pathogen. Clin Infect Dis **26:**1179–81.

[42] **Adhikari, R. P., and R. P. Novick.** 2008. Regulatory organization of the staphylococcal *sae* locus. Microbiology **154:**949–59.

[43] **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc Natl Acad Sci U S A **89:** 6462–66.

[44] **Fournier, B., and D. C. Hooper.** 2000. A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J Bacteriol **182:**3955–64.

[45] **Luong, T. T., S. W. Newell, and C. Y. Lee.** Mgr, a novel global regulator in *Staphylococcus aureus*. J Bacteriol **185:**3703-10.

[46] **Nicholas, R. O., T. Li, D. Mcdevitt, A. Marra, S. Sucoloski, P. L. Demarsh, and D. R. Gentry.** 1999. Isolation and characterization of a *sigB* deletion mutant of *Staphylococcus aureus.* Infect Immun **67:**3667-69.

[47] **Saïd-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan, and B. N. Kreiswirth.** 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J Bacteriol **185:**610–9.

[48] **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429–49.

[49] **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J **12:**3967–75.

[50] **Novick, R. P., and E. Geisinger.** 2008. Quorum sensing in staphylococci. Annu Rev Genet **42:**541–64.

[51] **Ji, G., R. Beavis, and R. P. Novick.** 1997. Bacterial interference caused by autoinducing peptide variants. Science **276:**2027–30.

[52] **Schweizer, M. L., J. P. Furuno, G. Sakoulas, J. K. Johnson, A. D. Harris, M. D. Shardell, J. C. McGregor, K. A. Thom, and E. N. Perencevich.** 2011. Increased mortality with accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* among bacteremic patients. Antimicrob Agents Chemother **55:**1082–87.

[53] **Peacock, S. J., C. E. Moore, A. Justice, M. Kantzanou, L. Story, K. Mackie, G. O'Neill, and N. P. Day. 2002.** Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect Immun **70:**4987–96.

[54] **Gordon, R. J., and F. D. Lowy.** 2008. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis **46:**S350–59.

[55] **Melles, D. C., R. F. Gorkink, H. A. Boelens, S. V. Snijders, J. K.**

Peeters, M. J. Moorhouse, P. J. van der Spek, W. B. van Leeuwen, G. Simons, H. A. Verbrugh, and A. van Belkum. 2004. Natural population dynamics and expansion of pathogenic clones of *Staphylococcus aureus*. J Clin Invest **114:**1732–40.

[56] **Patti, J. M., B. L. Allen, M. J. McGavin, and M. Höök.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol **48:**585–617.

[57] **Walsh, E. J., H. Miajlovic, O. V. Gorkun, and T. J. Foster.** 2008. Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the C-domain of human fibrinogen. Microbiology **154:**550–8.

[58] **Foster, T. J., J. A. Geoghegan, V. K. Ganesh, and M. Höök.** 2013. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus.* Nat Rev Microbiol **12:**49–62.

[59] **Chavakis, T., K. Wiechmann, K. T. Preissner, and M. Herrmann.** 2005. *Staphylococcus aureus* interactions with the endothelium: the role of bacterial "secretable expanded repertoire adhesive molecules" (SERAM) in disturbing host defense systems. Thromb Haemost **94:**278–85.

[60] **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms : Survival Mechanisms of Clinically Relevant Microorganisms. Clin Microbiol Rev **15:**167–93.

[61] **von Eiff, C.** 2008. *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. Int J Antimicrob Agents **31:** 507–10.

[62] **Proctor, R. A., C. von Eiff, B. C. Kahl, K. Becker, P. McNamara, M. Herrmann, and G. Peters.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol **4:**295–305.

[63] **Besier, S., C. Smaczny, C. von Mallinckrodt, A. Krahl, H. Ackermann, V. Brade, and T. A. Wichelhaus.** 2007. Prevalence and Clinical

Significance of *Staphylococcus aureus* Small-Colony Variants in Cystic Fibrosis Lung Disease. J Clin Microbiol **45:**168–72.

[64] **Tzianabos, A. O., J. Y. Wang, and J. C. Lee.** 2001. Structural rationale for the modulation of abscess formation by *Staphylococcus aureus* capsular polysaccharides. Proc Natl Acad Sci U S A **98:**9365–70.

[65] **O'Riordan, K., and J. Lee.** 2004. *Staphylococcus aureus* capsular polysaccharides. Clin Microbiol Rev **17:**218–34.

[66] **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat Rev Microbiol **3:**948–58.

[67] **Lee, H. W., P. W. Lee, and K. M. Johnson.** 2004. Isolation of the etiologic agent of Korean hemorrhagic fever. 1978. J Infect Dis **190:**1711–21.

[68] **Rooijakkers, S. H., K. P. van Kessel, and J. A. van Strijp.** 2005. Staphylococcal innate immune evasion. Trends Microbiol **13:**596–601.

[69] **Veldkamp, K. E., H. C. Heezius, J. Verhoef, J. A. van Strijp, and K. P. van Kessel.** 2000. Modulation of neutrophil chemokine receptors by *Staphylococcus aureus* supernate. Infect Immun **68:**5908–13.

[70] **de Haas, C. J., K. E. Veldkamp, A. Peschel, F. Weerkamp, W. J. van Wamel, E. C. Heezius, M. J. Poppelier, K. P. van Kessel, and J. A. van Strijp.** 2004. Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. J Exp Med **199:**687–95.

[71] **Haas, P. J., C. J. de Haas, W. Kleibeuker, M. J. Poppelier, K. P. van Kessel, J. A. Kruijtzer, R. M. Liskamp, and J. A. van Strijp. 2004.** "N-terminal residues of the chemotaxis inhibitory protein of *Staphylococcus aureus* are essential for blocking formylated peptide receptor but not C5a receptor. J Immunol **173:**5704–11.

[72] **Postma, B., M. J. Poppelier, J. C. van Galen, E. R. Prossnitz, J. A. van Strijp, C. J. de Haas, and K. P. van Kessel.** 2004. Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and

formylated peptide receptor. J Immunol **172:**6994–7001.

[73] **Postma, B., W. Kleibeuker, M. J. Poppelier, M. Boonstra, K. P. van Kessel, J. A. van Strijp, and C. J. de Haas.** 2005. Residues 10-18 within the C5a receptor N terminus compose a binding domain for chemotaxis inhibitory protein of *Staphylococcus aureus*. J Biol Chem **280:**2020–7.

[74] **Rooijakkers, S. H., M. Ruyken, A. Roos, M. R. Daha, J. S. Presanis, R. B. Sim, W. J. B. van Wamel, K. P. van Kessel, and J. A. van Strijp.** 2005. Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol **6:**920–7.

[75] **Rooijakkers, S. H., M. Ruyken, J. van Roon, K. P. van Kessel, J. A. van Strijp, and W. J. van Wamel.** 2006. Early expression of SCIN and CHIPS drives instant immune evasion by *Staphylococcus aureus.* Cell Microbiol **8:**1282–93.

[76] **Jin, H., R. Carrio, A. Yu, and T. R. Malek.** 2004. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. J Immunol **173:**657–65.

[77] **Rooijakkers, S. H., W. J. van Wamel, M. Ruyken, K. P. van Kessel, and J. A. van Strijp.** 2005. Anti-opsonic properties of staphylokinase. Microbes Infect **7:**476–84.

[78] **van Wamel, W. J., S. H. Rooijakkers, M. Ruyken, K. P. van Kessel, and J. A. van Strijp.** 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. **188:**1310–15.

[79] **McCarthy, A. J., W. van Wamel, S. Vandendriessche, J. Larsen, O. Denis, C. Garcia-Graells, A. C. Uhlemann, F. D. Lowy, R. Skov, and J. A. Lindsay.** 2012. *Staphylococcus aureus* CC398 clade associated with human-to-human transmission. Appl Environ Microbiol **78:**8845-8.

[80] **Price, L. B., M. Stegger, H. Hasman, M. Aziz, J. Larsen, S. Andersen, T. Pearson, A. E. Waters, J. T. Foster, J. Schupp, J. Gillece, E.**

Driebe, C. M. Liu, B. Springer, I. Zdovc, A. Battisti, A. Franco, J. Zmudzki, S. Schwarz, P. Butaye, E. Jouy, C. Pomba, M. C. Porrero, R. Ruimy, T. C. Smith, D. A. Robinson, J. S. Weese, C. S. Arriola, F. Yu, F. Laurent, P. Keim, R. Skov, and F. M. Aarestrup. 2012. *Staphylococcus aureus* CC398 : Host Adaptation and Emergence of Methicillin Resistance in Livestock. MBio. **3:**e00305–11.

[81] **Peschel, A., and M. Otto.** 2013. Phenol-soluble modulins and staphylococcal infection. Nat Rev Microbiol **11:**667–73.

[82] **Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo, and M. Otto.** 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med **13:**1510–14.

[83] **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert.** 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu Rev Microbiol **55:**77–104.

[84] **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piémont, N. Brousse, D. Floret, and J. Etienne.** 2002. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet **359:**753–9.

[85] **Kaneko, J., T. Kimura, S. Narita, T. Tomita, and Y. Kamio.** 1998. Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage φPVL carrying Panton-Valentine leukocidin genes. Gene **215:**57–67.

[86] **Wardenburg, J. B., T. Bae, M. Otto, F. R. DeLeo, and O. Schneewind.** 2007. Poring over pores: α-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat Med **13:**1405–6.

[87] **David, M. Z., and R. S. Daum.** 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical

consequences of an emerging epidemic. Clin Microbiol Rev **23:**616–87.

[88] **Woodin, A. M.** 1960. Purification of the two components of leucocidin from *Staphylococcus aureus*. Biochem J **75:**158–65.

[89] **Boyle-Vavra, S., and R. S. Daum.** 2007. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton–Valentine leukocidin. Lab Investig **87:**3–9.

[90] **Otto, M. 2010.** Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu Rev Microbiol **64:**143–62.

[91] **Rossney, A. S., A. C. Shore, P. M. Morgan, M. M. Fitzgibbon, B. O'Connell, and D. C. Coleman.** 2007. The emergence and importation of diverse genotypes of methicillin-resistant *Staphylococcus aureus* (MRSA) harboring the panton-valentine leukocidin gene (*pvl*) reveal that *pvl* is a poor marker for community-acquired MRSA strains in Ireland. J Clin Microbiol **45:**2554–63.

[92] **Voyich, J. M., M. Otto, B. Mathema, K. R. Braughton, A. R. Whitney, D. Welty, R. D. Long, D. W. Dorward, D. J. Gardner, G. Lina, B. N. Kreiswirth, and F. R. DeLeo.** 2006. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis **194:**1761–70.

[93] **Diep, B. A., A. M. Palazzolo-Ballance, P. Tattevin, L. Basuino, K. R. Braughton, A. R. Whitney, L. Chen, B. N. Kreiswirth, M. Otto, F. R. DeLeo, and H. F. Chambers.** 2008. Contribution of Panton-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. PLoS One **3:**e3198.

[94] **Lina, G., Y. Piémont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne.** 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis **29:**1128–32.

[95] **Monecke, S., G. Coombs, A. C. Shore, D. C. Coleman, P. Akpaka, M. Borg, H. Chow, M. Ip, L. Jatzwauk, D. Jonas, K. Kadlec, A. Kearns, F.**

Laurent, F. G. O'Brien, J. Pearson, A. Ruppelt, S. Schwarz, E. Scicluna, P. Slickers, H. L. Tan, S. Weber, and R. Ehricht. 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One **6:**e17936.

[96] **Mandell, G. L.** 1975. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal--leukocyte interaction. J Clin Invest **55:**561–6.

[97] **Park, B, V. Nizet, and G. Y. Liu.** 2008. Role of *Staphylococcus aureus* catalase in niche competition against *Streptococcus pneumoniae*. J Bacteriol **190:**2275–78.

[98] **Hynes, W. L., and S. L. Walton.** 2000. Hyaluronidases of Gram-positive bacteria. FEMS Microbiol Lett **183:**201–7.

[99] **Peterson, P. K., B. J. Wilkinson, Y. Kim, D. Schmeling, S. D. Douglas, P. G. Quie, and J. Verhoef.** 1978. The key role of peptidoglycan in the opsonization of *Staphylococcus aureus*. J Clin Invest **61:**597–609.

[100] **Dinges, M. M., P. M. Orwin, and P. M. Schlievert.** 2000. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev **13:**16–34.

[101] **Baker, M. D., and K. R. Acharya.** 2004. Superantigens: structure-function relationships. Int J Med Microbiol **293:**529–37.

[102] **Fraser, J. D., and T. Proft.** 2008. The bacterial superantigen and superantigen-like proteins. Immunol Rev **225:**226–43.

[103] **Le Loir, Y., F. Baron, and M. Gautier.** 2003. *Staphylococcus aureus* and food poisoning. Genet Mol Res **2:**63–76.

[104] **Novick, R. P.** 2003. Mobile genetic elements and bacterial toxinoses: The superantigen-encoding pathogenicity islands of *Staphylococcus aureus.* Plasmid **49:**93–105.

[105] **van Trijp, M. J., D. C. Melles, S. V. Snijders, H. F. Wertheim, H. A. Verbrugh, A. van Belkum, and W. J. van Wamel.** 2010. Genotypes, superantigen gene profiles, and presence of exfoliative toxin genes in

clinical methicillin-susceptible *Staphylococcus aureus* isolates. Diagn Microbiol Infect Dis **66:**222–4.

[106] **Holtfreter, S., D. Grumann, M. Schmudde, H. T. Nguyen, P. Eichler, B. Strommenger, K. Kopron, J. Kolata, S. Giedrys-Kalemba, I. Steinmetz, W. Witte, and B. M. Bröker.** 2007. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J Clin Microbiol **45:**2669–80.

[107] **Salgado-Pabón, W., L. Breshears, A. R. Spaulding, J. A. Merriman, C. S. Stach, A. R. Horswill, M. L. Peterson, and M. Schlievert.** 2013. Superantigens are critical for *Staphylococcus aureus* infective endocarditis, sepsis, and acute kidney injury. MBio **4:**1–9.

[108] **Nishifuji, K., M. Sugai, and M. Amagai.** 2008. Staphylococcal exfoliative toxins: "molecular scissors" of bacteria that attack the cutaneous defense barrier in mammals. J Dermatol Sci **49:**21–31.

[109] **Yamaguchi, T., K. Nishifuji, M. Sasaki, Y. Fudaba, M. Aepfelbacher, T. Takata, M. Ohara, H. Komatsuzawa, M. Amagai, and M. Sugai.** 2002. Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. Infect Immun **70:** 5835–45.

[110] **Sato, H., Y. Matsumori, T. Tanabe, H. Saito, A. Shimizu, and J. Kawano.** 1994. A new type of staphylococcal exfoliative toxin from a *Staphylococcus aureus* strain isolated from a horse with phlegmon. Infect Immun **62:**3780–85.

[111] **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington.** 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus.* Lancet **367:**731–9.

[112] **Diep, B. A., G. G. Stone, L. Basuino, C. J. Graber, A. Miller, S. A. des Etages, A. Jones, A. M. Palazzolo-Ballance, F. Perdreau-Remington,**

G. F. Sensabaugh, F. R. DeLeo, and H. F. Chambers. 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus.* J Infect Dis **197:**1523–30.

[113] **Tenover, F. C., and R. V. Goering.** 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. J Antimicrob Chemother **64:**441–6.

[114] **Diep, B. A., and M. Otto.** 2008. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol **16:**361–9.

[115] **Goering, R. V., L. K. McDougal, G. E. Fosheim, K. K. Bonnstetter, D. J. Wolter, and F. C. Tenover.** 2007. Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J Clin Microbiol **45:**1981–4.

[116] **Ellington, M. J., L. Yearwood, M. Ganner, C. East, and A. M. Kearns.** 2008. Distribution of the ACME-*arcA* gene among methicillin-resistant *Staphylococcus aureus* from England and Wales. J Antimicrob Chemother **61:**73–7.

[117] **Ellis, M. W., M. E. Griffith, J. H. Jorgensen, D. R. Hospenthal, K. Mende, and J. E. Patterson.** 2009. Presence and molecular epidemiology of virulence factors in methicillin-resistant *Staphylococcus aureus* strains colonizing and infecting soldiers. J Clin Microbiol **47:**940–5.

[118] **E. Ghaznavi-Rad, M. Nor Shamsudin, Z. Sekawi, L. Y. Khoon, M. N. Aziz, R. A. Hamat, N. Othman, P. P. Chong, A. van Belkum, H. Ghasemzadeh-Moghaddam, and V. Neela.** 2010. Predominance and emergence of clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* in Malaysia. J Clin Microbiol **48:**867–72.

[119] **Barbier, F., D. Lebeaux, D. Hernandez, A. S. Delannoy, V. Caro, P.**

François, J. Schrenzel, E. Ruppé, K. Gaillard, M. Wolff, S. Brisse, A. Andremont, and R. Ruimy. 2010. High prevalence of the arginine catabolic mobile element in carriage isolates of methicillin-resistant *Staphylococcus epidermidis*. J Antimicrob Chemother **66:**29–36.

[120] **Miragaia, M., H. de Lencastre, F. Perdreau-Remington, H. F. Chambers, J. Higashi, P. M. Sullam, J. Lin, K. I. Wong, K. A. King, M. Otto, G. F. Sensabaugh, and B. A. Diep.** 2009. Genetic diversity of arginine catabolic mobile element in *Staphylococcus epidermidis*. PLoS One **4:**e7722.

[121] **Pi, B., M. Yu, Y. Chen, Y. Yu, and L. Li.** 2009. Distribution of the ACME-*arcA* gene among meticillin-resistant *Staphylococcus haemolyticus* and identification of a novel *ccr* allotype in ACME-*arcA*-positive isolates. J Med Microbiol **58:**731–6.

[122] **McAdow, M., D. M. Missiakas, and O. Schneewind.** 2012. *Staphylococcus aureus* secretes coagulase and von willebrand factor binding protein to modify the coagulation cascade and establish host infections. J Innate Immu*n* **4:**141–8.

[123] **Cheng, A. G., M. McAdow, H. K. Kim, T. Bae, D. M. Missiakas, and O. Schneewind.** Contribution of coagulases towards *Staphylococcus aureus* disease and protective immunity. PLoS Pathog **6:**19–20.

[124] **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol Rev **59:**171–200.

[125] **Nascimento, J. S., J. Abrantes, M. Giambiagi-deMarval, and M. C. F. Bastos.** 2004. Growth conditions required for bacteriocin production by strains of *Staphylococcus aureu*s. World J Microbiol Biotechnol **20:**941–7.

[126] **Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki.** 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev **24:** 85–106.

[127] **McAuliffe, O., R. P. Ross, and C. Hill.** 2001. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev **25:**285–308.

[128] **Daly, K. M., M. Upton, S. K. Sandiford, L. A. Draper, P. A. Wescombe, R. W. Jack, P. M. O'Connor, A. Rossney, F. Götz, C. Hill, P. D. Cotter, R. P. Ross, and J. R. Tagg.** 2010. Production of the Bsa lantibiotic by community-acquired *Staphylococcus aureus* strains. J Bacteriol **192:**1131–42.

[129] **Skinner, D., and C. S. Keefer.** 1941. Significance of bacteremia caused by *Staphylococcus aureus*: a study of one hundred and twenty-two cases and a review of the literature concerned with experimental infection in animals. Arch Intern Med **68**: 851–75.

[130] **Chain, E., H. W. Florey, A. D. Gardner, N. G. Heatley, M. A. Jennings, J. Orr-Ewing, and A. G. Sanders.** 1940. Penicillin as a chemotherapeutic agent. Lancet **236:**226–8.

[131] **Kloos, W. E.** 1998. *Staphylococcus*, p. 577-632. *In* L. Collier, A. Balows, and M. Sussman (ed.)*,* Topley and Wilson's microbiology and microbial infections, 9th ed. Edward Arnold, London, UK.

[132] **Clatworthy, A. E., E. Pierson, and D. T. Hung.** 2007. Targeting virulence: a new paradigm for antimicrobial therapy. Nat Chem Biol **3:**541–8.

[133] **Smith, D. L., J. Dushoff, and J. G. Morris.** 2005. Agricultural Antibiotics and Human Health. PLoS Med **2:**e232.

[134] **Wright, G. D.** 2010. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol **8:**123.

[135] **Madigan, M., J. Martinko, and J. Parker.** 2000. Microbial growth control, p. 765-771. *In* P. F. Corey (ed.), Brock, biology of microorganisms, 9th ed. Prentice-Hall, Inc., New Jersey, USA.

[136] **Kehrenberg, C., and S. Schwarz.** 2006. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. Antimicrob Agents Chemother **50:**1156–63.

[137] **Chen, H. J., W. C. Hung, S. P. Tseng, J. C. Tsai, P. R. Hsueh, and L. J.**

Teng. 2010. Fusidic acid resistance determinants in *Staphylococcus aureus* clinical isolates. Antimicrob Agents Chemother **54:**4985–91.

[138] **Poole, K.** 2004. Resistance to beta-lactam antibiotics. Cell Mol Life Sci **61:** 2200–23.

[139] **Hartman, B. J., and A. Tomasz.** 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus.* J Bacteriol **158:**513–6.

[140] **Ba, X., E. M. Harrison, G. F. Edwards, M. T. Holden, A. R. Larsen, A. Petersen, R. L. Skov, S. J. Peacock, J. Parkhill, G. K. Paterson, and M. A. Holmes.** Novel mutations in penicillin-binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the mec gene. J Antimicrob Chemother **69:** 594–7.

[141] **Hackbarth, C. J., T. Kocagoz, S. Kocagoz, and H. F. Chambers.** 1995. Point mutations in *Staphylococcus aureus* PBP 2 gene affect penicillin-binding kinetics and are associated with resistance. Antimicrob Agents Chemother **39:**103–6.

[142] **Nadarajah, J., M. J. Lee, L. Louie, L. Jacob, A. E. Simor, M. Louie, and M. J. McGavin.** 2006. Identification of different clonal complexes and diverse amino acid substitutions in penicillin-binding protein 2 (PBP2) associated with borderline oxacillin resistance in Canadian *Staphylococcus aureus* isolates. J Med Microbiol **55:**1675–83.

[143] **Gál, Z., P. Kovács, F. Hernádi, G. Barabás, L. Kiss, A. Iglói, and I. Szabó.** 2001. Investigation of oxacillin-hydrolyzing beta-lactamase in borderline methicillin-resistant clinical isolates of *Staphylococcus aureus.* Chemotherapy **47:**233–8.

[144] **McDougal, L.K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol **41:**5113–20.

[145] **Tomasz, A., H. B. Drugeon, H. M. De Lencastre, D. Jabes, L. McDougall, and J. Bille.** New mechanism for methicillin resistance in *Staphylococcus aureus*: Clinical isolates that lack the PBP 2a gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob Agents Chemother **33:**1869–74.

[146] **Pinho, M.** 2008. Mechanisms of beta-lactams and glycopeptide resistance in *Staphylococcus aureus*, p. 207-226. *In* J. Lindsay (ed.), *Staphylococcus* molecular genetics, 1st ed., Caister Academic Press Norfolk, UK.

[147] **Lyon, B. R., and R. Skurray.** 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic Basis. Microbiol Rev **51:**88–134.

[148] **Malachowa, N., and F. R. DeLeo.** 2010. Mobile genetic elements of *Staphylococcus aureus.* Cell Mol Life Sci **67:**3057–71.

[149] **Zapun, A., C. Contreras-Martel, and T. Vernet.** 2008. Penicillin-binding proteins and β-lactam resistance. FEMS Microbiol Rev **32:**361–85.

[150] **Jevons, M. P.** 1961. Celbenin-resistant staphylococci. Br Med J **1:**124-5.

[151] **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc Natl Acad Sci U S A **99:**7687–92.

[152] **Grundmann, H., M. Aires-de-Sousa, J. Boyce, and E. Tiemersma.** 2006. Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet **368:**874–85.

[153] **Utsui, Y., and T. Yokota.** 1985. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus.* Antimicrob Agents Chemother **28:**397–403.

[154] **Song, M.D., M. Wachi, M. Doi, F. Ishino, and M. Matsuhashi.** 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett **221:**167–71.

[155] **Ito, T., Y. Katayama, and K. Hiramatsu.** 1999. Cloning and nucleotide

sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob Agents Chemother **43:**1449–58.

[156] **Katayama, Y., T. Ito, and K. Hiramatsu.** 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus.* Antimicrob Agents Chemother **44:**1549–55.

[157] **Couto, I., H. de Lencastre, E. Severina, W. Kloos, J. A. Webster, R. J. Hubner, I. S. Sanches, and A. Tomasz.** 1996. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri.* Microb Drug Resist **2:**377–91.

[158] **Wu, S., C. Piscitelli, H. de Lencastre, and A. Tomasz.** 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb Drug Resist **2:**435–41.

[159] **Kim, C., C. Milheirico, S. Gardete, M. A. Holmes, M. T. Holden, H. de Lencastre, and A. Tomasz.** 2012. Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain LGA251 and its contribution to the beta-lactam-resistant phenotype. J Biol Chem **287:**36854–63.

[160] **García-Álvarez, L., M. T. Holden, H. Lindsay, C. R. Webb, D. F. Brown, M. D. Curran, E. Walpole, K. Brooks, D. J. Pickard, C. Teale, J. Parkhill, S. D. Bentley, G. F. Edwards, E. K. Girvan, A. M. Kearns, B. Pichon, R. L. Hill, A. R. Larsen, R. L. Skov, S. J. Peacock, D. J. Maskell, and M. A. Holmes.** 2011. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis **11:**595–603.

[161] **Shore, A. C., A. S. Rossney, O. M. Brennan, P. M. Kinnevey, H. Humphreys, D. J. Sullivan, R. V. Goering, R. Ehricht, S. Monecke, and D. C. Coleman.** 2011. Characterization of a novel arginine catabolic mobile element (ACME) and staphylococcal chromosomal cassette *mec* composite island with significant homology to *Staphylococcus epidermidis*

ACME type II in methicillin-resistant Staphylococcus aureus genotype. Antimicrob Agents Chemother **55:**1896–1905.

[162] **Deplano, A., S. Vandendriessche, C. Nonhoff, and O. Denis.** 2014. Genetic diversity among methicillin-resistant *Staphylococcus aureus* isolates carrying the *mecC* gene in Belgium. J Antimicrob Chemother **69:**1457–60.

[163] **Becker, K., B. Ballhausen, R. Köck, and A. Kriegeskorte.** 2014. Methicillin resistance in *Staphylococcus isolates*: The "*mec* alphabet" with specific consideration of *mecC*, a mec homolog associated with zoonotic *S. aureus* lineages. Int J Med Microbiol **304:**794–804.

[164] **Paterson, G. K., E. M. Harrison, and M. A. Holmes.** 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus.* Trends Microbiol **22:**42–7.

[165] **Boundy, S., M. K. Safo, L. Wang, F. N. Musayev, H. C. O'Farrell, J. P. Rife, and G. L. Archer.** 2013. Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfx*, the gene containing the staphylococcal chromosome cassette *mec* (SCC*mec*) insertion site. J Biol Chem **288:**132–40.

[166] **Hiramatsu, K., K. Asada, E. Suzuki, K. Okonogi, and T. Yokota.** 1992. Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA). FEBS Lett **298:**133–6.

[167] **de Lencastre, H., D. Oliveira, and A. Tomasz.** 2007. Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. Curr Opin Microbiol **10:**428–35.

[168] **International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC).** 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother **53:**4961–7.

[169] **Arêde, P., T. Botelho, T. Guevara, I. Usón, D. C. Oliveira, and F. X. Gomis-Rüth.** 2013. Structure-function studies of the staphylococcal methicillin resistance antirepressor MecR2. J Biol Chem **288:**21267–78.

[170] **Arêde, P., C. Milheiriço, H. de Lencastre, and D. C. Oliveira.** 2012. The anti-repressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. PLoS Pathog **8:**e1002816.

[171] **Hanssen, A. M., and J. U. Ericson Sollid.** 2006. SCC *mec* in staphylococci: genes on the move. FEMS Immunol Med Microbiol **46:**8–20.

[172] **Ito, T., X. X. Ma, F. Takeuchi, K. Okuma, H. Yuzawa, and K. Hiramatsu.** 2004. Novel Type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob Agents Chemother **48:**2637–51.

[173] **Ito, T., Y. Katayama, K. Asada, N. Mori, K. Tsutsumimoto, C. Tiensasitorn, and K. Hiramatsu.** 2001. Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **45:**1323–36.

[174] **Ito, T., K. Okuma, X. X. Ma, H. Yuzawa, and K. Hiramatsu.** 2003. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Drug Resist Updat **6:**41–52.

[175] **Milheiriço, C., D. C. Oliveira, and H. de Lencastre.** 2007. Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: 'SCC*mec* IV multiplex'. J Antimicrob Chemother **60:**42–8.

[176] **Berglund, C., T. Ito, M. Ikeda, X. X. Ma, B. Söderquist, and K. Hiramatsu.** 2008. Novel type of staphylococcal cassette chromosome *mec* in a methicillin-resistant *Staphylococcus aureus* strain isolated in Sweden. Antimicrob Agents Chemother **52:**3512–16.

[177] **Chongtrakool, P., T. Ito, X. X. Ma, Y. Kondo, S. Trakulsomboon, C. Tiensasitorn, M. Jamklang, T. Chavalit, J. H. Song, and K. Hiramatsu.** 2006. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries : a proposal for a new nomenclature for SCC*mec* elements. Antimicrob Agents Chemother **50:**1001–12.

[178] **Kwon, N. H., K. T. Park, J. S. Moon, W. K. Jung, S. H. Kim, J. M. Kim, S. K. Hong, H. C. Koo, Y. S. Joo, and Y. H. Park.** 2005. Staphylococcal cassette chromosome *mec* (SCC*mec*) characterization and molecular analysis for methicillin-resistant *Staphylococcus aureus* and novel SCC*mec* subtype IVg isolated from bovine milk in Korea. J Antimicrob Chemother **56:**624–32.

[179] **X. X. Ma, T. Ito, C. Tiensasitorn, M. Jamklang, P. Chongtrakool, S. Boyle-Vavra, R. S. Daum, and K. Hiramatsu.** 2002. Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother **46:**1147–52.

[180] **Milheiriço, C., D. C. Oliveira, and H. de Lencastre.** 2007. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob Agents Chemother **51:**3374–7.

[181] **Oliveira, D.C., C. Milheiriço, and H. de Lencastre.** 2006. Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCC*mec* type VI. Antimicrob Agents Chemother **50:**3457–9.

[182] **Zhang, K., J. A. McClure, S. Elsayed, and J. M. Conly.** 2009. Novel staphylococcal cassette chromosome *mec* type, tentatively designated type VIII, harboring class A *mec* and type 4 *ccr* gene complexes in a Canadian epidemic strain of methicillin-resistant *Staphylococcus aureus.* Antimicrob Agents Chemother **53:**531-40.

[183] **French, G. L.** 2006. Bactericidal agents in the treatment of MRSA infections--the potential role of daptomycin. J Antimicrob Chemother **58:**1107–17.

[184] **Han, L. L., L. K. McDougal, R. J. Gorwitz, K. H. Mayer, J. B. Patel, J. M. Sennott, and J. L. Fontana.** 2007. High frequencies of clindamycin and tetracycline resistance in methicillin-resistant *Staphylococcus aureus* pulsed-field type USA300 isolates collected at a Boston ambulatory health center. J Clin Microbiol **45:**1350–2.

[185] **European Centre for Disease Prevention and Control**. Annual epidemiological report 2014. Antimicrobial resistance and healthcare-associated infections. Stockholm: ECDC; 2015.

[186] **Gardete, S., and A. Tomasz.** 2014. Mechanisms of vancomycin resistance in *Staphylococcus aureus*. J Clin Invest **124:**2836–40.

[187] **Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover.** 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J Antimicrob Chemother **40:**135–6.

[188] **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–3.

[189] **Ariza, J., M. Pujol, J. Cabo, C. Peña, N. Fernández, J. Liñares, J. Ayats, and F. Gudiol.** 1999. Vancomycin in surgical infections due to meticillin-resistant *Staphylococcus aureus* with heterogeneous resistance to vancomycin. Lancet **353:**1587–8.

[190] **Tenover, F. C., L. M. Weigel, P. C. Appelbaum, L. K. Mcdougal, J. Chaitram, S. Mcallister, N. Clark, G. Killgore, C. M. O'Hara, L. Jevitt, J. B. Patel, and B. Bozdogan.** 2004. Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. Antimicrob Agents Chemother **48:**275–80.

[191] **Loeffler, A., A. M. Kearns, M. J. Ellington, L. J. Smith, V. E. Unt, J. A. Lindsay, D. U. Pfeiffer, and D. H. Lloyd.** 2009. First isolation of MRSA ST398 from UK animals: a new challenge for infection control teams? J

Hosp Infect **72:**269–71.

[192] **Smith, T. L., M. L. Pearson, K. R. Wilcox, C. Cruz, M. V. Lancaster, B. Robinson-Dunn, F. C. Tenover, M. J. Zervos, J. D. Band, E. White, and W. R. Jarvis.** 1999. Emergence of vancomycin resistance in *Staphylococcus aureus.* N Engl J Med **340:**605–12.

[193] **Walsh T. R., and R. A. Howe.** 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus Aureus.* Annu Rev Microbiol **56:**657–75.

[194] **Holmes, N. E., P. D. Johnson, and B. P. Howden.** 2012. Relationship between vancomycin-resistant *Staphylococcus aureus*, vancomycin-intermediate *S. aureus*, high vancomycin MIC, and outcome in serious *S. aureus* infections. J Clin Microbiol **50:**2548–52.

[195] **Lowy, F.** 2003. Antimicrobial resistance: the example of *Staphylococcus aureus.* J Clin Invest **111:**1265–73.

[196] **González-Zorn, B., and P. Courvalin.** 2003. *vanA*-mediated high level glycopeptide resistance in MRSA. Lancet Infect Dis **3:**67–8.

[197] **Eisenstein, B. I., F. B. Oleson, and R. H. Baltz.** 2010. Daptomycin: from the mountain to the clinic, with essential help from Francis Tally, MD. Clin Infect Dis **50:**S10-5.

[198] **Pogliano, J., N. Pogliano, and J. A. Silverman.** 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol **194:**4494–504.

[199] **Bayer, A. S., T. Schneider, and H. G. Sahl.** 2014. Mechanisms of datptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. Ann N Y Acad Sci **1277:**139–58.

[200] **Kelley, W. L., D. P. Lew, and A. Renzoni.** 2012. Antimicrobial peptide exposure and reduced susceptibility to daptomycin: insights into a complex genetic puzzle. J Infect Dis **206:**1153–6.

[201] **Moise, P. A., D. North, J. N. Steenbergen, and G. Sakoulas.** 2009.

Susceptibility relationship between vancomycin and daptomycin in *Staphylococcus aureus*: facts and assumptions. Lancet Infect Dis **9:**617–24.

[202] **Clinical Laboratory Standards Institute.** 2012. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement M100-S22. Wayne, Pennsylvania.

[203] **Liu, C., A. Bayer, S. E. Cosgrove, R. S. Daum, S. K. Fridkin, R. J. Gorwitz, S. L. Kaplan, A. W. Karchmer, D. P. Levine, B. E. Murray, M. J. Rybak, D. A. Talan, and H. F. Chambers.** 2011. Clinical practice guidelines by the infectious diseases society of america for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children: executive summary. Clin Infect Dis **52:**285–92.

[204] **Hayden, M. K., K. Rezai, R. A. Hayes, K. Lolans, J. P. Quinn, and R. A. Weinstein.** 2005. Development of daptomycin resistance *in vivo* in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **43:**5285–7.

[205] **Hirschwerk, D., C. C. Ginocchio, M. Bythrow, and S. Condon.** 2006. Diminished susceptibility to daptomycin accompanied by clinical failure in a patient with methicillin-resistant *Staphylococcus aureus* bacteremia. Infect Control Hosp Epidemiol **27:**315–7.

[206] **Skiest, D. J.** 2006. Treatment failure resulting from resistance of *Staphylococcus aureus* to daptomycin. J Clin Microbiol **44:**655–6.

[207] **Sakoulas, G., W. Rose, M. J. Rybak, S. Pillai, J. Alder, R. C. Moellering, and G. M. Eliopoulos.** 2008. Evaluation of endocarditis caused by methicillin-susceptible *Staphylococcus aureus* developing nonsusceptibility to daptomycin. J Clin Microbiol **46:**220–4.

[208] **Friedman, L., J. D. Alder, and J. A. Silverman.** 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother **50:**2137–45.

[209] **Mwangi, M. M., S. W. Wu, Y. Zhou, K. Sieradzki, H. de Lencastre, P. Richardson, D. Bruce, E. Rubin, E. Myers, E. D. Siggia, and A.**

Tomasz. 2007. Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. Proc Natl Acad Sci U S A **104:**9451–6.

[210] **Ernst, C. M., P. Staubitz, N. N. Mishra, S. J. Yang, G. Hornig, H. Kalbacher, A. S. Bayer, D. Kraus, and A. Peschel.** 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog **5:**e1000660.

[211] **Oku, Y., K. Kurokawa, N. Ichihashi, and K. Sekimizu.** 2004. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology **150:**45–51.

[212] **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Götz.** 1999. Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem **274:**8405–10.

[213] **Neuhaus, F. C., and J. Baddiley.** 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev **67:**686–723.

[214] **Peleg, A, Y., S. Miyakis, D. V. Ward, A. M. Earl, A. Rubio, D. R. Cameron, S. Pillai, R. C. Moellering, and G. M. Eliopoulos.** 2012. Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus.* PLoS One **7:** e28316.

[215] **Bozdogan, B., and P. C. Appelbaum.** 2004. Oxazolidinones: activity, mode of action, and mechanism of resistance. Int J Antimicrob Agents **23:**113–9.

[216] **Livermore, D. M.** 2003. Linezolid *in vitro*: mechanism and antibacterial spectrum. J Antimicrob Chemother **51:**9ii–16.

[217] **Leach, K. L., S. M. Swaney, J. R. Colca, W. G. McDonald, J. R. Blinn, L. M. Thomasco, R. C. Gadwood, D. Shinabarger, L. Xiong, and A. S. Mankin.** 2007. The site of action of oxazolidinone antibiotics in living bacteria and in human mitochondria. Mol Cell **26:**393–402.

[218] **Swaney, S. M., H. Aoki, M. C. Ganoza, and D. L. Shinabarger.** 1998. The oxazolidinone linezolid inhibits initiation of protein synthesis in bacteria. Antimicrob Agents Chemother **42:**3251–5.

[219] **Morales, G., J. J. Picazo, E. Baos, F. J. Candel, A. Arribi, B. Peláez, R. Andrade, M. de la Torre, J. Fereres, and M. Sánchez-García.** 2010. Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus.* Clin Infect Dis **50:**821–5.

[220] **Gopegui, E. R., C. Juan, L. Zamorano, J. L. Pérez, and A. Oliver.** 2012. Transferable multidrug resistance plasmid carrying *cfr* associated with *tet*(L), *ant(4')-Ia*, and *dfrK* genes from a clinical methicillin-resistant *Staphylococcus aureus* ST125 strain. *Antimicrob Agents Chemother* **56:**2139–42.

[221] **Besier, S., A. Ludwig, J. Zander, V. Brade, and T. A. Wichelhaus.** 2008. Linezolid resistance in *Staphylococcus aureus*: Gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob Agents Chemother **52:**1570–2.

[222] **Locke, J. B., M. Hilgers, and K. J. Shaw.** 2009. Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in staphylococci of clinical origin. Antimicrob Agents Chemother **53:**5275–8.

[223] **Gao, W., K. Chua, J. K. Davies, H. J. Newton, T. Seemann, P. F. Harrison, N. E. Holmes, H. W. Rhee, J. I. Hong, E. L. Hartland, T. P. Stinear, and B. P. Howden.** 2010. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog **6:**e1000944.

[224] **Arias, C. A., M. Vallejo, J. Reyes, D. Panesso, J. Moreno, E. Castañeda, M. V Villegas, B. E. Murray, and J. P. Quinn.** 2008. Clinical and microbiological aspects of linezolid resistance mediated by the *cfr* gene encoding a 23S rRNA methyltransferase. J Clin Microbiol **46:**892–6.

[225] **Schwarz, S., C. Werckenthin, and C. Kehrenberg.** 2000. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother **44:**2530–3.

[226] **Kehrenberg, C., S. Schwarz, L. Jacobsen, L. H. Hansen, and B. Vester.** 2005. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. Mol Microbiol **57:**1064–73.

[227] **Toh, S. M., L. Xiong, C. A. Arias, M. V Villegas, K. Lolans, J. Quinn, and A. S. Mankin.** 2007. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol **64:**1506–14.

[228] **Freire-Moran, L., B. Aronsson, C. Manz, I. C. Gyssens, A. D. So, D. L. Monnet, and O. Carsm; ECDC-EMA Working Group.** 2011. Critical shortage of new antibiotics in development against multidrug-resistant bacteria - Time to react is now. Drug Resist Updat **14:**118–24.

[229] **Proctor, R. A.** 2012. Is there a future for a *Staphylococcus aureus* vaccine? Vaccine **30:**2921–7.

[230] **Daum, R. S., and B. Spellberg.** 2012. Progress toward a *Staphylococcus aureu*s vaccine. Clin Infect Dis **54:**560–7.

[231] **Waldor, M. K.** 2006. Disarming pathogens - A new approach for antibiotic development. N Engl J Med **354:**296–7.

[232] **Sun, H., Y. Xu, I. Sitkiewicz, Y. Ma, X. Wang, B. D. Yestrepsky, Y. Huang, M. C. Lapadatescu, M. J. Larsen, S. D. Larsen, J. M. Musser, and D. Ginsburg.** 2012. Inhibitor of streptokinase gene expression improves survival after group A streptococcus infection in mice. Proc Natl Acad Sci U S A **109:**3469–74.

[233] **Rasko, D. A., C. G. Moreira, R. de Li, N. C. Reading, J. M. Ritchie, M. K. Waldor, N. Williams, R. Taussig, S. Wei, M. Roth, D. T. Hughes, J. F. Huntley, M. W. Fina, J. R. Falck, and V. Sperandio.** 2008. Targeting QseC signaling and virulence for antibiotic development. Science **321:**1078–80.

[234] **Ma, Y., Y. Xu, B. D. Yestrepsky, R. J. Sorenson, M. Chen, S. D. Larsen, and H. Sun.** 2012. Novel inhibitors of *Staphylococcus aureus* virulence gene expression and biofilm formation. PLoS One **7:**e47255.

[235] **Merckoll, P., T. Ø. Jonassen, M. E. Vad, S. L. Jeansson, and K. K. Melby.** 2009. Bacteria, biofilm and honey: a study of the effects of honey on 'planktonic' and biofilm-embedded chronic wound bacteria. Scand J Infect Dis **41:**341–7.

[236] **Haste, N. M., C. C. Hughes, D. N. Tran, W. Fenical, P. R. Jensen, V. Nizet, and M. E. Hensler.** Pharmacological properties of the marine natural product marinopyrrole A against methicillin-resistant *Staphylococcus aureus.* Antimicrob Agents Chemother **55:**3305–12.

[237] **Deurenberg, R. H., and E. E. Stobberingh.** 2008. The evolution of *Staphylococcus aureus*. Infect Genet Evol **8:**747–63.

[238] **Klevens, R. M., M. A. Morrison, S. K. Fridkin, A. Reingold, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, G. Fosheim, L. K. McDougal, and F. C. Tenover; Active Bacterial Core Surveillance of the Emerging Infectious Program Network.** 2006. Community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors. Emerg Infect Dis **12:**1991–3.

[239] **Otter, J. A. and G. L. French.** 2011. Community-associated meticillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J Hosp Infect **79:**189–93.

[240] **Krziwanek, K., S. Metz-Gercek, and H. Mittermayer.** 2009. Methicillin-resistant *Staphylococcus aureus* ST398 from human patients, upper

Austria. Emerg Infect Dis **15:**766–9.

[241] **Pan, A., A. Battisti, A. Zoncada, F. Bernieri, M. Boldini, A. Franco, M. Giorgi, M. Lurescia, S. Lorenzotti, M. Martinotti, M. Monaci, and A. Pantosti.** 2009. Community-acquired methicillin-resistant *Staphylococcus aureus* ST398 infection, Italy. Emerg Infect Dis **15:**845–7.

[242] **van Belkum, A., D. C. Melles, J. K. Peeters, W. B. van Leeuwen, E. van Duijkeren, X. W. Huijsdens, E. Spalburg, A. J. de Neeling, and H. A. Verbrugh; Dutch Working Party on Surveillance and Research of MRSA-SOM.** 2008. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg Infect Dis **14:**479–83.

[243] **van Loo, I., X. Huijsdens, E. Tiemersma, A. de Neeling, N. van de Sande-Bruinsma, D. Beaujean, A. Voss, and J. Kluytmans.** 2007. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg Infect Dis **13:**1834–9.

[244] **Witte, W., B. Strommenger, C. Stanek, and C. Cuny.** 2007. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg Infect Dis **13:**255–8.

[245] **Wulf, M. W., A. Markestein, F. T. van der Linden, A. Voss, C. Klaassen, and C. M. Verdurin.** 2008. First outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June 2007. Eurosurveillance **13:**pii:8051.

[246] **Fitzgerald, J. R.** 2012. Human origin for livestock-associated methicillin-resistant *Staphylococcus aureus*. MBio **3:**e00082–12.

[247] **Schijffelen, M. J., C. H. Boel, J. A. van Strijp, and A. C. Fluit.** 2010. Whole genome analysis of a livestock-associated methicillin-resistant *Staphylococcus aureus* ST398 isolate from a case of human endocarditis. BMC Genomics **11:**376.

[248] **Mole, B.** 2013. MRSA: Farming up trouble. Nature **499:**398–400.

[249] **Groves, M. D., M. V. O'Sullivan, H. J. Brouwers, T. A. Chapman, S. Abraham, D. J. Trott, R. Al Jassim, G. W. Coombs, R. L. Skov, D. Jordan.** 2014. *Staphylococcus aureus* ST398 detected in pigs in Australia. J Antimicrob Chemother **69:**1426–8.

[250] **Gomes, A. R., H. Westh, and H. De Lencastre.** 2006. Origins and evolution of methicillin-resistant *Staphylococcus aureus* clonal lineages. Antimicrob Agents Chemother **50:**3237–44.

[251] **Nübel, U., P. Roumagnac, M. Feldkamp, J. H. Song, K. S. Ko, Y. C. Huang, G. Coombs, M. Ip, H. Westh, R. Skov, M. J. Struelens, R. V Goering, B. Strommenger, A. Weller, W. Witte, and M. Achtman.** 2008. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A **105:**14130–5.

[252] **Kreiswirth, B., J. Kornblum, R. D. Arbeit, W. Eisner, J. N. Maslow, A. McGeer, D. E. Low, and R. P. Novick.** 1993. Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. Science **259:**227–30.

[253] **Crisóstomo, M. I., H. Westh, A. Tomasz, M. Chung, D. C. Oliveira, and H. de Lencastre.** 2001. The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proc Natl Acad Sci U S A **98:**9865–70.

[254] **Aires de Sousa, M., and H. de Lencastre.** 2004. Bridges from hospitals to the laboratory: Genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. FEMS Immunol Med Microbiol **40:**101–11.

[255] **Witte, W., M. Kresken, C. Braulke, and C. Cuny.** 1997. Increasing incidence and widespread dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals in central Europe, with special reference to German hospitals. Clin Microbiol Infect **3:**414–22.

[256] **Wannet, W. J., E. Spalburg, M. E. Heck, G. N. Pluister, R. J. Willems, and A. J. de Neeling.** 2004. Widespread dissemination in The

Netherlands of the epidemic berlin methicillin-resistant *Staphylococcus aureus* clone with low-level resistance to oxacillin. J Clin Microbiol **42:**3077–82.

[257] **Moore, P. C., and J. A. Lindsay.** 2002. Molecular characterisation of the dominant UK methicillin-resistant *Staphylococcus aureus* strains, EMRSA-15 and EMRSA-16. J Med Microbiol **51:**516–21.

[258] **Johnson, A. P., A. Pearson, and G. Duckworth.** 2005. Surveillance and epidemiology of MRSA bacteraemia in the UK. J Antimicrob Chemother **56:**455–62.

[259] **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus.* Antimicrob Agents Chemother **47:**3926–34.

[260] **Robinson D. A., and M. C. Enright.** 2004. Evolution of *Staphylococcus aureus* by large chromosomal replacements. J Bacteriol **186:**1060–4.

[261] **Deurenberg, R. H., E. Nulens, H. Valvatne, S. Sebastian, C. Driessen, J. Craeghs, E. De Brauwer, B. Heising, Y. J. Kraat, J. Riebe, F. S. Stals, T. A. Trienekens, J. Scheres, A. W. Friedrich, F. H. van Tiel, P. S. Beisser, and E. E. Stobberingh.** 2009. Cross-border dissemination of methicillin-resistant *Staphylococcus aureus*, Euregio Meuse-Rhin region. Emerg Infect Dis **15:**727–34.

[262] **Conceição, T., M. Aires-de-sousa, M. Füzi, Á. Tóth, J. Pászti, E. Ungvári, W. B. van Leeuwen, A. van Belkum, H. Grundmann, and H. de Lencastre.** 2007. Replacement of methicillin-resistant *Staphylococcus aureus* clones in Hungary over time: A 10-year surveillance study. Clin Microbiol Infect **13:**971–9.

[263] **Shore A., A. S. Rossney, C. T. Keane, M. C. Enright, and D. C. Coleman.** 2005. Seven novel variants of the staphylococcal chromosomal cassette *mec* in methicillin-resistant *Staphylococcus aureus* isolates from Ireland. Antimicrob Agents Chemother **49:**2070–83.

[264] **Aires-de-Sousa, M., B. Correia, and H. de Lencastre; Multilaboratory**

Project Collaborators. 2008. Changing patterns in frequency of recovery of five methicillin-resistant *Staphylococcus aureus* clones in Portuguese hospitals: surveillance over a 16-year period. J Clin Microbiol **46:**2912–17.

[265] **Campanile, F., D. Bongiorno, S. Borbone, and S. Stefani.** 2009. Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. Ann Clin Microbiol Antimicrob **8:**22.

[266] **Robinson, D. A., A. M. Kearns, A. Holmes, D. Morrison, H. Grundmann, G. Edwards, F. G. O'Brien, F. C. Tenover, L. K. McDougal, A. B. Monk, and M. C. Enright.** 2005. Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. Lancet **365:**1256–8.

[267] **Guggenbichler, J. P., O. Assadian, M. Boeswald, and A. Kramer.** 2011. Incidence and clinical implication of nosocomial infections associated with implantable biomaterials - catheters, ventilator-associated pneumonia, urinary tract infections. GMS Krankenhhyg Interdiszip **6:**Doc18.

[268] **Chavez, T. T., and C. F. Decker.** 2008. Health care-associated MRSA versus community-associated MRSA. Dis Mon **54:**763–8.

[269] **Boucher, H. W., and G. R. Corey.** 2008. Epidemiology of methicillin-resistant *Staphylococcus aureus.* Clin Infect Dis **46:**S344–S349.

[270] **Mera, R. M., J. A. Suaya, H. Amrine-Madsen, C. S. Hogea, L. A. Miller, E. P. Lu, D. F. Sahm, P. O'Hara, and C. J. Acosta.** 2011. Increasing role of *Staphylococcus aureus* and community-acquired methicillin-resistant *Staphylococcus aureus* infections in the United States: a 10-year trend of replacement and expansion. Microb Drug Resist **17:**321–8.

[271] **Cuevas, O., E. Cercenado, M. J. Goyanes, A. Vindel, P. Trincado, T. Boquete, M. Marín, and E. Bouza; Grupo Español para el Estudio de Estafilococo.** 2008. *Staphylococcus spp*. en España: Situación actual y

evolución de la resistencia a antimicrobianos (1986-2006). Enferm Infecc Microbiol Clin **26:**269–77.

[272] **Falagas, M. E., D. E. Karageorgopoulos, J. Leptidis, and I. P. Korbila.** 2013. MRSA in Africa: filling the global map of antimicrobial resistance. PLoS One. **8:**e68024.

[273] **Chen, C. J., and Y. C. Huang.** 2014. New epidemiology of *Staphylococcus aureus* infection in Asia. Clin Microbiol Infect **20:**605–23.

[274] **Bell, J. M., J. D. Turnidge, and SENTRY APAC.** 2002. High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY antimicrobial surveillance program, 1998-1999. Antimicrob Agents Chemother **46:**879–81.

[275] **Deurenberg, R. H., and E. E. Stobberingh.** 2009. The molecular evolution of hospital- and community-associated methicillin-resistant *Staphylococcus aureus*. Curr Mol Med **9:**100–15.

[276] **Deurenberg, R. H., C. Vink, S. Kalenic, A. W. Friedrich, C. A. Bruggeman, and E. E. Stobberingh.** 2007. The molecular evolution of methicillin-resistant *Staphylococcus aureus.* Clin Microbiol Infect **13:**222-35.

[277] **Otter J. A., and G. L. French.** 2010. Molecular epidemiology of community-associated meticillin-resistant *Staphylococcus aureus* in Europe. Lancet Infect Dis **10:**227–39.

[278] **Coelho, C., C. Torres, H. Radhouani, L. Pinto, C. Lozano, E. Gómez-Sanz, M. Zaragaza, G. Igrejas, and P. Poeta.** 2011. Molecular detection and characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from dogs in Portugal. Microb Drug Resist **17:**333–7.

[279] **Horner, C., P. Parnell, D. Hall, A. Kearns, J. Heritage, and M. Wilcox.** 2013. Meticillin-resistant *Staphylococcus aureus* in elderly residents of care homes: colonization rates and molecular epidemiology. J Hosp Infect **83:**212–8.

[280] **Vandendriessche, S., W. Vanderhaeghen, F. V. Soares, M. Hallin, B. Catry, K. Hermans, P. Butaye, F. Haesebrouck, M. J. Struelens, and O. Denis.** 2013. Prevalence, risk factors and genetic diversity of methicillin-resistant *Staphylococcus aureus* carried by humans and animals across livestock production sectors. J Antimicrob Chemother **68:**1510–6.

[281] **Simões, R. R., M. Aires-de-Sousa, T. Conceição, F. Antunes, P. M. da Costa, and H. de Lencastre.** 2011. High prevalence of EMRSA-15 in Portuguese public buses: a worrisome finding. PLoS One **6:**e17630.

[282] **Conceição, T., F. Diamantino, C. Coelho, H. de Lencastre, and M. Aires-de-Sousa.** 2013. Contamination of public buses with MRSA in Lisbon, Portugal: a possible transmission route of major MRSA clones within the community. PLoS One **8:**e77812.

[283] **Bonura, C., M. R. Plano, P. Di Carlo, C. Calà, D. Cipolla, G. Corsello, C. Mammina, and EPI-MRSA Working Group.** 2010. MRSA ST22-IVa (EMRSA-15 clone) in Palermo, Italy. J Infect Public Health **3:**188–91.

[284] **Ozaki, K., M. Takano, W. Higuchi, T. Takano, S. Yabe, Y. Nitahara, A. Nishiyama, and T. Yamamoto.** 2009. Genotypes, intrafamilial transmission, and virulence potential of nasal methicillin-resistant *Staphylococcus aureus* from children in the community. J Infect Chemother **15:**84–91.

[285] **Khokhlova, O., Y. Tomita, W. C. Hung, T. Takano, Y. Iwao, W. Higuchi, A. Nishiyama, I. Reva, and T. Yamamoto.** 2015. Elderly infection in the community due to ST5/SCC*mec*II methicillin-resistant *Staphylococcus aureus* (the New York/Japan clone) in Japan: Panton-Valentine leukocidin-negative necrotizing pneumonia. J Microbiol Immunol Infect **48:**335–9.

[286] **Iwao, Y., S. Yabe, T. Takano, W. Higuchi, A. Nishiyama, and T. Yamamoto.** 2012. Isolation and molecular characterization of methicillin-resistant *Staphylococcus aureus* from public transport. Microbiol Immunol **56:**76–82.

[287] **Song, J. H., P. R. Hsueh, D. R. Chung, K. S. Ko, C.-I. Kang, K. R. Peck, J. S. Yeom, S. W. Kim, H. H. Chang, Y. S. Kim, S. I. Jung, J. S. Son, T. M. So, M. K. Lalitha, Y. Yang, S. G. Huang, H. Wang, Q. Lu, C. C. Carlos, J. A. Perera, C. H. Chiu, J. W. Liu, A. Chongthaleong, V. Thamlikitkul, and P. H. Van; ANSORP Study Group.** 2011. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. J Antimicrob Chemother **66:**1061–9.

[288] **Centers for Disease Control and Prevention**. 1999. Ten great public health achievements--United States, 1900-1999. JAMA **281:**1481.

[289] **Gorak, E. J., S. M. Yamada, and J. D. Brown.** 1999. Community-acquired methicillin-resistant *Staphylococcus aureus* in hospitalized adults and children without known risk factors. Clin Infect Dis **29:**797–800.

[290] **Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum.** 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA **279:**593–8.

[291] **Udo, E. E., J. W. Pearman, and W. B. Grubb.** 1993. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J Hosp Infect **25:**97–108.

[292] **Centers for Disease Control and Prevention.** 1999. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*- Minnesota and North Dakota, 1997-1999. JAMA **282:**1123-5.

[293] **Salgado, C. D., B. M. Farr, and D. P. Calfee.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus*: a meta-analysis of prevalence and risk factors. Clin Infect Dis **36:**131–9.

[294] **Naimi, T. S., K. H. LeDell, K. Como-Sabetti, S. M. Borchardt, D. J. Boxrud, J. Etienne, S. K. Johnson, F. Vandenesch, S. Fridkin, C. O'Boyle, R. N. Danila, and R. Lynfield.** 2003. Comparison of community- and health care-associated methicillin-resistant

Staphylococcus aureus infection. JAMA **290:**2976–84.

[295] **Miller, L. G., S. J. Eells, A. R. Taylor, M. Z. David, N. Ortiz, D. Zychowski, N. Kumar, D. Cruz, S. Boyle-Vavra, and R. S. Daum.** 2012. *Staphylococcus aureus* colonization among household contacts of patients with skin infections: risk factors, strain discordance, and complex ecology. Clin Infect Dis **54:**1523–35.

[296] **Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu.** 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J Clin Microbiol, **40:**4289–94.

[297] **Takano, T., W. Higuchi, and T. Yamamoto.** 2009. Superior *in vitro* activity of carbapenems over anti-methicillin-resistant *Staphylococcus aureus* (MRSA) and some related antimicrobial agents for community-acquired MRSA but not for hospital-acquired MRSA. J Infect Chemother **15:**54–7.

[298] **O'Brien, F. G., T. T. Lim, F. N. Chong, G. W. Coombs, M. C. Enright, D. A. Robinson, A. Monk, B. Saïd-Salim, B. N. Kreiswirth, and W. B. Grubb.** 2004. Diversity among community isolates of methicillin-resistant *Staphylococcus aureus* in Australia. J Clin Microbiol **42:**3185–90.

[299] **Diep, B. A., L. Chan, P. Tattevin, O. Kajikawa, T. R. Martin, L. Basuino, T. T. Mai, H. Marbach, K. R. Braughton, A. R. Whitney, D. J. Gardner, X. Fan, C. W. Tseng, G. Y. Liu, C. Badiou, J. Etienne, G. Lina, M. A. Matthay, F. R. DeLeo, and H. F. Chambers.** 2010. Polymorphonuclear leukocytes mediate *Staphylococcus aureus* Panton-Valentine leukocidin-induced lung inflammation and injury. Proc Natl Acad Sci U S A **107:**5587–92.

[300] **Gillet, Y., P. Vanhems, G. Lina, M. Bes, F. Vandenesch, D. Floret, and J. Etienne.** 2007. Factors predicting mortality in necrotizing community-acquired pneumonia caused by *Staphylococcus aureus* containing Panton-Valentine leukocidin. Clin Infect Dis **45:**315–21.

[301] **Zhang, K., J. A. McClure, S. Elsayed, J. Tan, and J. M. Conly.** 2008. Coexistence of Panton-Valentine leukocidin-positive and -negative community-associated methicillin-resistant *Staphylococcus aureus* USA400 sibling strains in a large Canadian health-care region. J Infect Dis **197:**195–204.

[302] **Li, M., B. A. Diep, A. E. Villaruz, K. R. Braughton, X. Jiang, F. R. DeLeo, H. F. Chambers, Y. Lu, and M. Otto.** 2009. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A **106:**5883–8.

[303] **Aires de Sousa, M., and H. de Lencastre.** 2003. Evolution of sporadic isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and their similarities to isolates of community-acquired MRSA. J Clin Microbiol **41:**3806–15.

[304] **Monecke, S., P. Slickers, M. J. Ellington, A. M. Kearns, and R. Ehricht.** High diversity of Panton–Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus.* Clin Microbiol Infect **13:**1157–64.

[305] **DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers.** 2010. Community-associated meticillin-resistant *Staphylococcus aureus.* Lancet **375:**1557–68.

[306] **Vandenesch F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne.** 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-valentine leukocidin genes: worldwide emergence. Emerg Infect Dis **9:**978–84.

[307] **Coombs, G. W., J. C. Pearson, F. G. O'Brien, R. J. Murray, W. B. Grubb, and K. J. Christiansen.** 2006. Methicillin-resistant *Staphylococcus aureus* clones, Western Australia. Emerg Infect Dis **12:**241–7.

[308] **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan; Emergency ID Net Study Group.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **355:**666–74.

[309] **David, M. Z., J. D. Siegel, H. F. Chambers, and R. S. Daum.** 2008. Determining whether methicillin-resistant *Staphylococcus aureus* is associated with health care. JAMA **299:**519.

[310] **Zetola, N., J. S. Francis, E. L. Nuermberger, and W. R. Bishai.** 2005. Community-acquired meticillin-resistant *Staphylococcus aureus*: An emerging threat. Lancet Infect Dis **5:**275–86.

[311] **Seybold, U., E. V Kourbatova, J. G. Johnson, S. J. Halvosa, Y. F. Wang, M. D. King, S. M. Ray, and H. M. Blumberg.** 2006. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin Infect Dis **42:**647–56.

[312] **Nimmo, G. R.** 2012. USA300 abroad: Global spread of a virulent strain of community-associated methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect **18:**725–34.

[313] **Rolo, J., M. Miragaia, A. Turlej-Rogacka, J. Empel, O. Bouchami, N. A. Faria, A. Tavares, W. Hryniewicz, A. C. Fluit, and H. de Lencastre.** 2012. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One **7:**e34768.

[314] **Boyle-vavra, S., B. Ereshefsky, C. C. Wang, and R. S. Daum.** 2005. Successful multiresistant community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel Staphylococcal chromosome cassette *mec* (SCC*mec*) type VT or SCC*mec* type IV. J Clin Microbiol **43:**4719–30.

[315] **Wang, C. C., W. T. Lo, M. L. Chu, and L. K. Siu.** 2004. Epidemiological typing of community-acquired methicillin-resistant *Staphylococcus aureus*

isolates from children in Taiwan. Clin Infect Dis **39:**481–7.

[316] **Takano, T., W. Higuchi, T. Otsuka, T. Baranovich, S. Enany, K. Saito, H. Isobe, S. Dohmae, K. Ozaki, M. Takano, Y. Iwao, M. Shibuya, T. Okubo, S. Yabe, D. Shi, I. Reva, L. J. Teng, and T. Yamamoto.** 2008. Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* strains belonging to multilocus sequence type 59 in Taiwan. Antimicrob Agents Chemother **52:**837–45.

[317] **Ho, P. L., C. Cheung, G. C. Mak, C. W. Tse, T. K. Ng, C. H. Cheung, T. L. Que, R. Lam, R. W. Lai, R. W. Yung, and K. Y. Yuen.** 2007. Molecular epidemiology and household transmission of community-associated methicillin-resistant *Staphylococcus aureus* in Hong Kong. Diagn Microbiol Infect Dis **57:**145–51.

[318] **Tenover, F. C., L. K. McDougal, R. V Goering, G. Killgore, S. J. Projan, J. B. Patel, and P. M. Dunman.** 2006. Characterization of a strain of community- associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J Clin Microbiol **44:**108–18.

[319] **Aires de Sousa, M., C. Bartzavali, I. Spiliopoulou, I. Santos Sanches, M. I. Crisóstomo, and H. de Lencastre.** 2003. Two international methicillin-resistant *Staphylococcus aureus* clones endemic in a University Hospital in Patras, Greece. J Clin Microbiol **41:**2027–32.

[320] **Bekkhoucha, S. N., A. Cady, P. Gautier, F. Itim, and P. Y. Donnio.** A portrait of *Staphylococcus aureus* from the other side of the Mediterranean Sea: molecular characteristics of isolates from Western Algeria. Eur J Clin Microbiol Infect Dis **28:**553–5.

[321] **Faria, N. A., D. C. Oliveira, H. Westh, D. L. Monnet, A. R. Larsen, R. Skov, and H. de Lencastre.** 2005. Epidemiology of emerging methicillin-resistant *Staphylococcus aureus* (MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. J Clin Microbiol **43:**1836–42.

[322] **Denis, O., A. Deplano, H. De Beenhouwer, M. Hallin, G. Huysmans, M. G. Garrino, Y. Glupczynski, X. Malaviolle, A. Vergison, and M. J. Struelens.** 2005. Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium. J Antimicrob Chemother **56:**1103–6.

[323] **Faria, N. A., J. A. Carriço, D. C. Oliveira, M. Ramirez, and H. de Lencastre.** 2008. Analysis of typing methods for epidemiological surveillance of both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J Clin Microbiol **46:**136–44.

[324] **Manzur, A. M. A. Dominguez, M. Pujol, M. P. M. González, E. Limon, A. Hornero, R. Martín, F. Gudiol, and J. Ariza.** Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. Clin Microbiol Infect **14:**377–80.

[325] **Vindel, A., P. Trincado, O. Cuevas, C. Ballesteros, E. Bouza, and E. Cercenado.** 2014. Molecular epidemiology of community-associated methicillin-resistant *Staphylococcus aureus* in Spain: 2004-12. J Antimicrob Chemother **69:**2913-9.

[326] **Popovich, K. J., B. Hota, A. Aroutcheva, L. Kurien, J. Patel, R. Lyles-Banks, A. E. Grasso, A. Spec, K. G. Beavis, M. K. Hayden, and R. A. Weinstein.** 2013. "Community-associated methicillin-resistant *Staphylococcus aureus* colonization burden in HIV-infected patients. Clin Infect Dis **56:**1067–74.

[327] **Imaz, A., M. Camoez, S. Di Yacovo, O. Gasch, M. A. Dominguez, A. Vila, M. Maso-Serra, M. Pujol, and D. Podzamczer.** 2015. Prevalence of methicillin-resistant *Staphylococcus aureus* colonization in HIV-infected patients in Barcelona, Spain: a cross-sectional study. BMC Infect Dis **15:** 243.

[328] **Otter, J. A., and G. L. French.** 2012. Community-associated meticillin-resistant *Staphylococcus aureus*: the case for a genotypic definition. J Hosp Infect **81:**143–8.

[329] **Millar, B. C., A. Loughrey, J. S. Elborn, and J. E. Moore.** 2007. Proposed definitions of community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA). J Hosp Infect **67:**109-13.

[330] **van Cleef, B. A., D. L. Monnet, A. Voss, K. Krziwanek, F. Allerberger, M. Struelens, H. Zemlickova, R. L. Skov, J. Vuopio-Varkila, C. Cuny, A. W. Friedrich, I. Spiliopoulou, J. Pászti, H. Hardardottir, A. Rossney, A. Pan, A. Pantosti, M. Borg, H. Grundmann, M. Mueller-Premru, B. Olsson-Liljequist, A. Widmer, S. Harbarth, A. Schweiger, S. Unal, and J. A. Kluytmans.** 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis **17:**502–5.

[331] **Smith, T. C., M. J. Male, A. L. Harper, J. S. Kroeger, G. P. Tinkler, E. D. Moritz, A. W. Capuano, L. A. Herwaldt, and D. J. Diekema.** 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One 4:e4258.

[332] **Yu, F., Z. Chen, C. Liu, X. Zhang, X. Lin, S. Chi, T. Zhou, and X. Chen.** 2008. Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin Microbiol Infect **14:**381–4.

[333] **Golding, G. R., L. Bryden, P. N. Levett, R. R. McDonald, A. Wong, J. Wylie, M. R. Graham, S. Tyler, G. Van Domselaar, A. E. Simor, D. Gravel, and M. R. Mulvey.** 2010. Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 in humans, Canada. Emerg Infect Dis **16:**587–94.

[334] **van der Mee-Marquet, N. L., A. Corvaglia, M. Haenni, X. Bertrand, J. B. Franck, J. Kluytmans, M. Girard, R. Quentin, and P. François.** 2014. Emergence of a novel subpopulation of CC398 *Staphylococcus aureus* infecting animals is a serious hazard for humans. Front Microbiol **5:**652.

[335] **Stegger, M., J. A. Lindsay, M. Sørum, K. A. Gould, and R. Skov.** 2010. Genetic diversity in CC398 methicillin-resistant *Staphylococcus aureus*

isolates of different geographical origin. Clin Microbiol Infect **16:**1017–9.

[336] **Kadlec, K., R. Ehricht, S. Monecke, U. Steinacker, H. Kaspar, J. Mankertz, and S. Schwarz.** 2009. Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. *J Antimicrob Chemother* **64:**1156–64.

[337] **Cavaco, L. M., H. Hasman, and F. M. Aarestrup.** 2011. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. Vet Microbiol **150:**344–8.

[338] **Argudín, M. A., A. Fetsch, B. A. Tenhagen, J. A. Hammerl, S. Hertwig, J. Kowall, M. R. Rodicio, A. Käsbohrer, R. Helmuth, A. Schroeter, M. C. Mendoza, J. Bräunig, B. Appel, and B. Guerra.** 2010. High heterogeneity within methicillin-resistant *Staphylococcus aureus* ST398 isolates, defined by *Cfr9I* macrorestriction-pulsed-field gel electrophoresis profiles and *spa* and SCC*mec* types. Appl Environ Microbiol **76:**652–8.

[339] **Voss, A., F. Loeffen, J. Bakker, C. Klaassen, and M. Wulf.** 2005. “Methicillin-resistant *Staphylococcus aureus* in Pig Farming,” Emerg Infect Dis **11:**1965–6.

[340] **Garcia-Graells, C., J. Antoine, J. Larsen, B. Catry, R. Skov, and O. Denis.** 2012. Livestock veterinarians at high risk of acquiring methicillin-resistant *Staphylococcus aureus* ST398. Epidemiol Infect **140:**383–9.

[341] **Graveland, H., J. A. Wagenaar, K. Bergs, H. Heesterbeek, and D. Heederik.** 2011. Persistence of livestock associated MRSA CC398 in humans is dependent on intensity of animal contact. PLoS One **6:**e16830.

[342] **Köck, R., B. Loth, M. Köksal, J. Schulte-Wülwer, J. Harlizius, and A. W. Friedrich.** 2012. Persistence of nasal colonization with livestock-associated methicillin-resistant *Staphylococcus aureus* in pig farmers after holidays from pig exposure. Appl Environ Microbiol **78:**4046–7.

[343] **Lozano, C., A. Rezusta, P. Gómez, E. Gómez-Sanz, N. Báez, G. Martin-Saco, M. Zarazaga, and C. Torres.** 2012. 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 **67:**330–4.

[344] **Hallin, M., R. De Mendonça, O. Denis, A. Lefort, F. El Garch, P. Butaye, K. Hermans, and M. J. Struelens.** 2011. Diversity of accessory genome of human and livestock-associated ST398 methicillin resistant *Staphylococcus aureus* strains. Infect Genet Evol **11:**290–9.

[345] **Ekkelenkamp, M. B., M. Sekkat, N. Carpaij, A. Troelstra, and M. J. Bonten.** 2006. [Endocarditis due to meticillin-resistant Staphylococcus aureus originating from pigs]. Ned Tijdschr Geneeskd **150:**2442–7.

[346] **Armand-Lefevre, L., R. Ruimy, and A. Andremont.** 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg Infect Dis **11:**711–4.

[347] **Aspiroz, C., C. Lozano, A. Vindel, J. J. Lasarte, M. Zarazaga, and C. Torres.** 2010. Skin lesion caused by ST398 and ST1 MRSA, Spain. Emerg Infect Dis **16:**157–9.

[348] **Singh, A., R. V Goering, S. Simjee, S. L. Foley, and M. J. Zervos.** 2006. Application of molecular techniques to the study of hospital infection. Clin Microbiol Rev **19:**512–30.

[349] **Stepán, J., R. Pantůcek, and J. Doskar.** 2004. Molecular diagnostics of clinically important staphylococci. Folia Microbiol (Praha) **49:**353–86.

[350] **Friedrich, A. W., W. Witte, H. De Lencastre, W. Hryniewicz, J. Scheres, and H. Westh; SeqNet.org participants.** 2008. A European laboratory network for sequence-based typing of methicillin-resistant *Staphylococcus aureus* (MRSA) as a communication platform between human and veterinary medicine--an update on SeqNet.org. Euro Surveill **13:**pii:18862.

[351] **Struelens, M. J., P. M. Hawkey, G. L. French, W. Witte, and E. Tacconelli.** 2009. Laboratory tools and strategies for methicillin-resistant *Staphylococcus aureus* screening, surveillance and typing: state of the art and unmet needs. Clin Microbiol Infect **15:**112–9.

[352] **van Belkum, A., P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, S. Brisse, and M. Struelens; European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group on Epidemiological Markers (ESGEM).** 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect **13:**1–46.

[353] **Lina, G., F. Boutite, A. Tristan, M. Bes, J. Etienne, and F. Vandenesch.** Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. Appl Environ Microbiol **69:**18–23.

[354] **Monecke, S., L. Jatzwauk, S. Weber, P. Slickers, and R. Ehricht.** 2008. DNA microarray-based genotyping of methicillin-resistant *Staphylococcus aureus* strains from Eastern Saxony. Clin Microbiol Infect **14:**534–45.

[355] **Wolters, M., H. Rohde, T. Maier, C. Belmar-Campos, G. Franke, S. Scherpe, M. Aepfelbacher, and M. Christner.** 2011. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol **301:**64–8.

[356] **Josten, M., M. Reif, C. Szekat, N. Al-Sabti, T. Roemer, K. Sparbier, M. Kostrzewa, H. Rohde, H. G. Sahl, and G. Bierbaum.** 2013. Analysis of the matrix-assisted laser desorption ionization-time of flight mass spectrum of *Staphylococcus aureus* identifies mutations that allow differentiation of the main clonal lineages. J Clin Microbiol **51:**1809–17.

[357] **Boggs, S. R., L. H. Cazares, and R. Drake.** 2012. Characterization of a *Staphylococcus aureus* USA300 protein signature using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Med Microbiol **61:**640–4.

[358] **Schwartz, D. C., and C. R. Cantor.** 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell **37:**67–75.

[359] **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol **33:**2233–9.

[360] **Tenover, F. C., R. D. Arbeit, and R. V Goering.** 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect Control Hosp Epidemiol **18:**426–39.

[361] **Cookson, B.D., D. A. Robinson, A. B. Monk, S. Murchan, A. Deplano, R. de Ryck, M. J. Struelens, C. Scheel, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, C. Cuny, W. Witte, P. T. Tassios, N. J. Legakis, W. van Leeuwen, A. van Belkum, A. Vindel, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, M. Muller-Premru, W. Hryniewicz, A. Rossney, B. O'Connell, B. D. Short, J. Thomas, S. O'Hanlon, and M. C. Enright.** 2007. Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection. J Clin Microbiol **45:**1830–7.

[362] **Chung, M., H. De Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Domínguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sá-Leão, I. Santos Sanches, J. Song, P. T. Tassios, and P. Villari; Multilaboratory Project Collaborators.** 2000. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis : comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb Drug Resist **6:**189-98.

[363] **Murchan, S., M. E. Kaufmann, A. Deplano, R. de Ryck, M. Struelens, C. E. Zinn, V. Fussing, S. Salmenlinna, J. Vuopio-Varkila, N. El Solh, C. Cuny, W. Witte, P. T. Tassios, N. Legakis, W. van Leeuwen, A. van**

Belkum, A. Vindel, I. Laconcha, J. Garaizar, S. Haeggman, B. Olsson-Liljequist, U. Ransjo, G. Coombes, and B. Cookson. 2003. 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 **41:**1574–85.

[364] **Van Belkum, A., W. van Leeuwen, M. E. Kaufmann, B. Cookson, F. Forey, J. Etienne, R. Goering, F. Tenover, C. Steward, F. O'Brien, W. Grubb, P. Tassios, N. Legakis, A. Morvan, N. El Solh, R. de Ryck, M. Struelens, S. Salmenlinna, J. Vuopio-Varkila, M. Kooistra, A. Talens, W. Witte, and H. Verbrugh.** 1998. Assessment of resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed-field gel electrophoresis of SmaI macrorestriction fragments: A multicenter study. J Clin Microbiol **36:**1653–9.

[365] **Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A **95:**3140–5.

[366] **Enright, M. C., and B. G. Spratt.** 1999. Multilocus sequence typing. Trends Microbiol **7:**482–7.

[367] **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol **38:**1008–15.

[368] **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518–30.

[369] **Day, N. P., C. E. Moore, M. C. Enright, A. R. Berendt, J. M. Smith, M.**

F. Murphy, S. J. Peacock, B. G. Spratt, and E. J. Feil. 2001. A link between virulence and ecological abundance in natural populations of *Staphylococcus aureus*. Science **292:**114–6.

[370] **Francisco, A. P., M. Bugalho, M. Ramirez, and J. A. Carriço.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics **18;**10:152.

[371] **Frénay, H. M., A. E. Bunschoten, L. M. Schouls, W. J. van Leeuwen, C. M. Vandenbroucke-Grauls, J. Verhoef, and F. R. Mooi.** 1996. Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. Eur J Clin Microbiol Infect Dis **15:**60–4.

[372] **Guss, B., M. Uhlén, B. Nilsson, M. Lindberg, J. Sjöquist, and J. Sjödahl.** 1984. Region X, the cell-wall-attachment part of staphylococcal protein A. Eur J Biochem **138:**413–20.

[373] **Aires-de-Sousa, M., T. Conceição, and H. de Lencastre.** 2006. Unusually high prevalence of nosocomial Panton-Valentine leukocidin-positive *Staphylococcus aureus* isolates in Cape Verde Islands. J Clin Microbiol **44:**3790–3.

[374] **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol **37:**3556–63.

[375] **Kahl, B. C., A. Mellmann, S. Deiwick, G. Peters, and D. Harmsen.** 2005. Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. J Clin Microbiol **43:**502–5.

[376] **Harmsen, D., H. Claus, W. Witte, J. Rothgänger, H. Claus, D. Turnwald, and U. Vogel.** 2003. 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 **41:**5442–8.

[377] **Koreen, L., S. V Ramaswamy, E. A. Graviss, S. Naidich, J. M. Musser, and B. N. Kreiswirth.** 2004. *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J Clin Microbiol **42:**792–9.

[378] **Aires-de-Sousa, M., K. Boye, H. de Lencastre, A. Deplano, M. C. Enright, J. Etienne, A. Friedrich, D. Harmsen, A. Holmes, X. W. Huijsdens, A. M. Kearns, A. Mellmann, H. Meugnier, J. K. Rasheed, E. Spalburg, B. Strommenger, M. J. Struelens, F. C. Tenover, J. Thomas, U. Vogel, H. Westh, J. Xu, and W. Witte.** 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J Clin Microbiol 44:619–21.

[379] **Friedrich, A. W., W. Witte, D. Harmsen, H. de Lencastre, W. Hryniewicz, J. Scheres, H. Westh, and SeqNet.org participants.** 2006. SeqNet.org: a European laboratory network for sequence-based typing of microbial pathogens. Euro Surveill **11:**E060112.4.

[380] **Mellmann, A., T. Weniger, C. Berssenbrügge, J. Rothgänger, M. Sammeth, J. Stoye, and D. Harmsen.** 2007. Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. BMC Microbiol **7:**98.

[381] **Hallin, M., N. Maes, B. Byl, F. Jacobs, Y. De Gheldre, and M. J. Struelens.** 2003. Clinical impact of a PCR assay for identification of *Staphylococcus aureus* and determination of methicillin resistance directly from blood cultures. J Clin Microbiol **41:**3942–4.

[382] **Strommenger, B., C. Braulke, D. Heuck, C. Schmidt, B. Pasemann, U. Nübel, and W. Witte.** 2008. *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J Clin Microbiol **46:**574–81.

[383] **Strommenger, B., C. Kettlitz, T. Weniger, D. Harmsen, A. W.**

Friedrich, and W. Witte. 2006. Assignment of *Staphylococcus* isolates to groups by *spa* typing, *SmaI* macrorestriction analysis, and multilocus sequence typing. J Clin Microbiol **44:**2533–40.

[384] **Morfeldt, E., L. Janzon, S. Arvidson, and S. Löfdahl.** 1988. Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in Staphylococcus aureus. Mol Gen Genet **211:**435–40.

[385] **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick.** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar. Mol Gen Genet **202:**58–61.

[386] **Balaban, N., and R. P. Novick.** 1995. Autocrine regulation of toxin synthesis by *Staphylococcus aureus.* Proc Natl Acad Sci U S A **92:**1619–23.

[387] **Otto, M., R. Süssmuth, C. Vuong, G. Jung, and F. Götz.** 1999. Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis agr* pheromone and derivatives. FEBS Lett **450:**257–62.

[388] **Read, A. F., and L. H. Taylor.** 2001. The ecology of genetically diverse infections. Science **292:**1099–102.

[389] **Shopsin, B., B. Mathema, P. Alcabes, B. Said-Salim, G. Lina, A. Matsuka, J. Martinez, and B. N. Kreiswirth.** 2003. Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J Clin Microbiol **41:**456–9.

[390] **Oliveira, D. C., and H. de Lencastre.** 2002. Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus.* Antimicrob. Agents Chemother*.* **46:**2155–61.

[391] **Boye, K., M. D. Bartels, I. S. Andersen, J. A. Møller, and H. Westh.** 2007. A new multiplex PCR for easy screening of methicillin-resistant *Staphylococcus aureus* SCC*mec* types I-V. Clin Microbiol Infect **13:**725–7.

[392] **Kondo, Y., T. Ito, X. X. Ma, S. Watanabe, B. N. Kreiswirth, J. Etienne, and K. Hiramatsu.** 2007. Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob. Agents Chemother **51:**264–74.

[393] **Zhang, K., J. A. McClure, S. Elsayed, T. Louie, and J. M. Conly.** 2005. 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 **43:**5026–33.

[394] **Lina, G., G. Durand, C. Berchich, B. Short, H. Meugnier, F. Vandenesch, J. Etienne, and M. C. Enright.** 2006. Staphylococcal chromosome cassette evolution in *Staphylococcus aureus* inferred from *ccr* gene complex sequence typing analysis. Clin Microbiol Infect **12:**1175–84.

[395] **Oliveira, D. C., C. Milheiriço, S. Vinga, and H. de Lencastre.** 2006. Assessment of allelic variation in the *ccrAB* locus in methicillin-resistant *Staphylococcus aureu*s clones. J Antimicrob Chemother **58:**23–30.

[396] **Oliveira, D. C., M. Santos, C. Milheiriço, J. A. Carriço, S. Vinga, A. L. Oliveira, and H. De Lencastre.** 2008. *ccrB* typing tool: an online resource for staphylococci *ccrB* sequence typing. J Antimicrob Chemother **61:**959–60.

[397] **Turlej, A., W. Hryniewicz, and J. Empel.** 2011. Staphylococcal Cassette chromosome *mec* (SCCmec) classification and typing methods: an overview. Polish J Microbiol **60:**95–103.

[398] **Li, S., R. L. Skov, X. Han, A. R. Larsen, J. Larsen, M. Sørum, M. Wulf, A. Voss, K. Hiramatsu, and T. Ito.** 2011. Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus* strains. Antimicrob Agents Chemother **55:**3046–50.

[399] **Sabat, A. J., A. Budimir, D. Nashev, R. Sá-Leão, J. van Dijl, F.**

Laurent, H. Grundmann, and A. W. Friedrich; ESCMID Study Group of Epidemiological Markers (ESGEM). 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill **18:**20380.

[400] **Monecke, S., P. Slickers, and R. Ehricht.** 2008. Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol **53:**237–51.

[401] **Kurt, K., A. Alderborn, M. Nilsson, B. Strommenger, W. Witte, and U. Nübel.** 2009. Multiplexed genotyping of methicillin-resistant *Staphylococcus aureus* isolates by use of padlock probes and tag microarrays. J Clin Microbiol **47:**577–85.

[402] **Lindsay, J. A., C. E. Moore, N. P. Day, S. J. Peacock, A. A. Witney, R. A. Stabler, S. E. Husain, P. D. Butcher, and J. Hinds.** 2006. Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. J Bacteriol **188:**669–76.

[403] **Koessler, T., P. Francois, Y. Charbonnier, A. Huyghe, M. Bento, S. Dharan, G. Renzi, D. Lew, S. Harbarth, D. Pittet, and J. Schrenzel.** 2006. Use of oligoarrays for characterization of community-onset methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **44:**1040–8.

[404] **Zhu, L. X., Z. W. Zhang, C. Wang, H. W. Yang, D. Jiang, Q. Zhang, K. Mitchelson, and J. Cheng.** 2007. Use of a DNA microarray for simultaneous detection of antibiotic resistance genes among staphylococcal clinical isolates. J Clin Microbiol **45:**3514–21.

[405] **Jackson, K. A., V. Edwards-Jones, C. W. Sutton, and A. J. Fox.** 2005. Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J Microbiol Methods **62:**273–84.

[406] **Chen, J. H., W. C. Yam, A. H. Ngan, A. M. Fung, W. L. Woo, M. K. Yan, G. K. Choi, P. L. Ho, V. C. Cheng, and K. Y. Yuen.** 2013. Advantages of

using matrix-assisted laser desorption ionization-time of flight mass spectrometry as a rapid diagnostic tool for identification of yeasts and mycobacteria in the clinical microbiological laboratory. J Clin Microbiol **51:** 3981–7.

[407] **Carbonnelle, E., J. L. Beretti, S. Cottyn, G. Quesne, P. Berche, X. Nassif, and A. Ferroni.** 2007. Rapid identification of Staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **45:**2156–61.

[408] **Nagy, E., T. Maier, E. Urban, G. Terhes, M. Kostrzewa, and ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria.** 2009. Species identification of clinical isolates of *Bacteroides* by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. Clin Microbiol Infect **15:**796-802.

[409] **Rajakaruna, L., G. Hallas, L. Molenaar, D. Dare, H. Sutton, V. Encheva, R. Culak, I. Innes, G. Ball, A. M. Sefton, M. Eydmann, A. M. Kearns, and H. N. Shah.** 2009. High throughput identification of clinical isolates of *Staphylococcus aureus* using MALDI-TOF-MS of intact cells. Infect Genet Evol **9:**507–13.

[410] **Christner, M., H. Rohde, M. Wolters, I. Sobottka, K. Wegscheider, and M. Aepfelbacher.** 2010. Rapid identification of bacteria from positive blood culture bottles by use of matrix-assisted laser desorption-ionization time of flight mass spectrometry fingerprinting. J Clin Microbiol **48:**1584–91.

[411] **Seng P., J. M. Rolain, P. E. Fournier, B. La Scola, M. Drancourt, and R. Didier.** 2010. MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol **5:**1733–54.

[412] **Clark, A. E., E. J. Kaleta, A. Arora, and D. M. Wolk.** 2013. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev **26:**547–603.

[413] **Espinal, P., H. Seifert, L. Dijkshoorn, J. Vila, and I. Roca.** 2012. Rapid and accurate identification of genomic species from the *Acinetobacter baumannii* (Ab) group by MALDI-TOF MS. Clin Microbiol Infect **18:**1097–103.

[414] **Dieckmann, R., R. Helmuth, M. Erhard, and B. Malorny.** 2008. Rapid classification and identification of Salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol **74:**7767–78.

[415] **Jadhav, S., D. Sevior, M. Bhave, and E. A. Palombo.** 2014. Detection of *Listeria monocytogenes* from selective enrichment broth using MALDI-TOF Mass Spectrometry. J Proteomics **97:**100–6.

[416] **Ikryannikova, L., A. V. Filimonova, M. V. Malakhova, T. Savinova, O. Filimonova, E. Ilina, V. A. Dubovickaya, S. V. Sidorenko, and V. M. Govorun.** 2013. Discrimination between *Streptococcus pneumoniae* and *Streptococcus mitis* based on sorting of their MALDI mass spectra. Clin Microbiol Infect **19:**1066–71.

[417] **Edwards-jones, V., M. A. Claydon, D. J. Evason, J. Walker, A. J. Fox, and D. B. Gordon.** 2000. Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol **49:**295–300.

[418] **Schlebusch, S., G. R. Price, S. Hinds, C. Nourse, J. M. Schooneveldt, M. H. Tilse, H. G. Liley, T. Wallis, F. Bowling, D. Venter, and G. R. Nimmo.** 2010. First outbreak of PVL-positive nonmultiresistant MRSA in a neonatal ICU in Australia: comparison of MALDI-TOF and SNP-plus-binary gene typing. Eur J Clin Microbiol Infect Dis **29:**1311–4.

[419] **Wang, L., C. Han, W. Sui, M. Wang, and X. Lu.** 2013. MALDI-TOF MS applied to indirect carbapenemase detection: a validated procedure to clearly distinguish between carbapenemase-positive and carbapenemase-negative bacterial strains. Anal Bioanal Chem **405:**5259–66.

[420] **Gagnaire, J., O. Dauwalder, S. Boisset, D. Khau, A. M. Freydière, F. Ader, M. Bes, G. Lina, A. Tristan, M. E. Reverdy, A. Marchand, T. Geissmann, Y. Benito, G. Durand, J. P. Charrier, J. Etienne, M. Welker, A. Van Belkum, and F. Vandenesch.** 2012. Detection of *Staphylococcus aureus* delta-toxin production by whole-cell MALDI-TOF mass spectrometry. PLoS One **7:**e40660.

[421] **Lasch, P., C. Fleige, M. Stämmler, F. Layer, U. Nübel, W. Witte, and G. Werner.** 2014. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of *Enterococcus faecium* and *Staphylococcus aureus* isolates. J Microbiol Methods **100:**58–69.

[422] **Traber, K., and R. Novick.** 2006. A slipped-mispairing mutation in AgrA of laboratory strains and clinical isolates results in delayed activation of *agr* and failure to translate δ- and α-haemolysins. Mol Microbiol **59:**1519–30.

[423] **Francisco, A. P., C. Vaz, P. T. Monteiro, J. Melo-Cristino, M. Ramirez, and J. A. Carriço.** 2012. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics **13:**87.

[424] **Geha, D. J., J. R. Uhl, C. A. Gustaferro, and D. H. Persing.** 1994. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J Clin Microbiol **32:**1768–72.

[425] **Mick, V., M. A. Domínguez, F. Tubau, J. Liñares, M. Pujol, and R. Martín.** 2010. Molecular characterization of resistance to Rifampicin in an emerging hospital-associated Methicillin-resistant *Staphylococcus* aureus clone ST228, Spain. BMC Microbiol **10:**68.

[426] **B. Heym, M. Le Moal, L. Armand-Lefevre, and M. H. Nicolas-Chanoine.** 2002. Multilocus sequence typing (MLST) shows that the 'Iberian' clone of methicillin-resistant *Staphylococcus aureus* has spread to France and acquired reduced susceptibility to teicoplanin. J Antimicrob Chemother **50:**323–9.

[427] **Oliveira, D. C., A. Tomasz, and H. de Lencastre.** 2001. The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated mec elements. Microb Drug Resist **7:**349–61.

[428] **Monecke, S., and R. Ehricht.** 2005. Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. Clin Microbiol Infect **11:**825–33.

[429] **Stephens, M.** 1974. EDF Statistics for Goodness of Fit and Some Comparisons. J Am Stat Assoc **69:**730–7.

[430] **Rodríguez-Baño J., M. Angeles Domínguez, A. Blas Millán, C. Borraz, M. Pau González, B. Almirante, E. Cercenado, B. Padilla and M. Pujol; GEIH/GEMARA (SEIMC); REIPI Collaborators.** 2009. Clinical and molecular epidemiology of community-acquired, healthcare-associated and nosocomial methicillin-resistant *Staphylococus aureu*s in Spain. Clin Microbiol Infect **15:**1111–8.

[431] **Vindel, A., O. Cuevas, E. Cercenado, C. Marcos, V. Bautista, C. Castellares, P. Trincado, T. Boquete, M. Pérez-Vázquez, M. Marín, E. Bouza, and Spanish Group for the Study of *Staphylococcus*.** 2009. Methicillin-resistant *Staphylococcus aureus* in Spain: Molecular epidemiology and utility of different typing methods. J Clin Microbiol **47:**1620–7.

[432] **Wenzel, R. P., M. D. Nettleman, R. N. Jones, and M. A. Pfaller.** 1991. Methicillin-resistant *Staphylococcus aureus*: implications for the 1990s and effective control measures. Am J Med **91:**221S–227S.

[433] **European Centre for Disease Prevention and Control.** Antimicrobial resistance surveillance in Europe 2009. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2010.

[434] **Wertheim, H. F., M. C. Vos, H. A. Boelens, A. Voss, C. M. Vandenbroucke-Grauls, M. H. Meester, J. A. Kluytmans, P. H. van**

Keulen, and H. A. Verbrugh. 2004. Low prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) at hospital admission in the Netherlands: the value of search and destroy and restrictive antibiotic use. J Hosp Infect **56:**321–5.

[435] **Dominguez, M. A., H. de Lencastre, J. Linares, and A. Tomasz.** 1994. Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. J Clin Microbiol **32:**2081-7.

[436] **Oliveira, D., I. Santos-Sanches, R. Mato, M. Tamayo, G. Ribeiro, D. Costa, and H. de Lencastre.** 1998. Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the 'Iberian' and the 'Brazilian' clones of MRSA. Clin Microbiol Infect **4:**373–84.

[437] **Amorim, M. L., N. A. Faria, D. C. Oliveira, C. Vasconcelos, J. C. Cabeda, A. C. Mendes, E. Calado, A. P. Castro, M. H. Ramos, J. M. Amorim, and H. de Lencastre.** 2007. Changes in the clonal nature and antibiotic resistance profiles of methicillin-resistant *Staphylococcus aureus* isolates associated with spread of the EMRSA-15 clone in a tertiary care Portuguese hospital. J Clin Microbiol **45:**2881-8.

[438] **Denis, O., A. Deplano, R. De Ryck, C. Nonhoff, and M. J. Struelens.** 2003. Emergence and spread of gentamicin-susceptible strains of methicillin-resistant *Staphylococcus aureus* in Belgian hospitals. Microb Drug Resist **9:**61–71.

[439] **Donnio, P. Y., L. Preney, A. L. Gautier-Lerestif, J. L. Avril, and N. Lafforgue.** 2004. Changes in staphylococcal cassette chromosome type and antibiotic resistance profile in methicillin-resistant *Staphylococcus aureus* isolates from a French hospital over an 11 year period. J Antimicrob Chemother **53:**808–13.

[440] **Montesinos, I., T. Delgado, D. Riverol, E. Salido, M. A. Miguel, A. Jimenez, and A. Sierra.** 2006. Changes in the epidemiology of meticillin-resistant *Staphylococcus aureus* associated with the emergence of

EMRSA-16 at a university hospital. J Hosp Infect **64:**257–63.

[441] **Vindel, A., P. Trincado, E. Gómez, R. Cabrera, T. Boquete, C. Solá, S. Valdezate, and J. A. Saez-Nieto.** 2006. Prevalence and evolution of methicillin-resistant *Staphylococcus aureus* in Spanish hospitals between 1996 and 2002. J Clin Microbiol **44:**266–70.

[442] **Witte, W., C. Braulke, C. Cuny, D. Heuck, and M. Kresken.** 2001. Changing pattern of antibiotic resistance in methicillin-resistant *Staphylococcus aureus* from German hospitals. Infect Control Hosp Epidemiol **22:**683–6.

[443] **Laurent, F., H. Lelièvre, M. Cornu, F. Vandenesch, G. Carret, J. Etienne, and J. P. Flandrois.** 2001. Fitness and competitive growth advantage of new gentamicin-susceptible MRSA clones spreading in French hospitals. J Antimicrob Chemother **47:**277–83.

[444] **Cuevas, O., E. Cercenado, A. Vindel, J. Guinea, M. Sánchez-Conde, M. Sánchez-Somolinos, and E. Bouza.** 2004. Evolution of the antimicrobial resistance of *Staphylococcus spp*. in Spain: five nationwide prevalence studies, 1986 to 2002. Antimicrob Agents Chemother **48:**4240–5.

[445] **Ariza-Miguel, J., M. Hernández, I. Fernández-Natal, and D. Rodríguez-Lázaro.** 2014. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a university hospital in northwestern Spain. Int Microbiol **17:**149–57.

[446] **Lozano, C., N. Porres-Osante, J. Crettaz, B. Rojo-Bezares, D. Benito, I. Olarte, M. Zarazaga, Y. Sáenz and C. Torres.** 2013. Changes in genetic lineages, resistance, and virulence in clinical methicillin-resistant *Staphylococcus aureus* in a Spanish hospital. J Infect Chemother **19:**233–42.

[447] **Schuenck, R. P., S. A. Nouér, C. de Oliveira Winter, F. S. Cavalcante, T. D. Scotti, A. L. Ferreira, M. Giambiagi-de Marval, and K. R. Netto dos Santos.** 2009. Polyclonal presence of non-multiresistant methicillin-

resistant *Staphylococcus aureus* isolates carrying SCC*mec* IV in health care-associated infections in a hospital in Rio de Janeiro, Brazil. Diagn Microbiol Infect Dis **64:**434–41.

[448] **Cuevas, O., E. Cercenado, E. Bouza, C. Castellares, P. Trincado, R. Cabrera, and A. Vindel; Spanish Group for the Study of *Staphylococcus*.** 2007. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Spain: a multicentre prevalence study (2002). Clin Microbiol Infect **13:**250–6.

[449] **Rossney, A., and S. O'Connell.** 2008. Emerging high-level mupirocin resistance among MRSA isolates in Ireland. Euro Surveill **13:**pi:8084.

[450] **Walker, E. S., F. Levy, M. Shorman, G. David, J. Abdalla, and F. A. Sarubbi.** 2004. A decline in mupirocin resistance in methicillin-resistant *Staphylococcus aureus* accompanied administrative control of prescriptions. J Clin Microbiol **42:**2792–5.

[451] **Appelbaum, P. C.** 2007. Reduced glycopeptide susceptibility in methicillin-resistant *Staphylococcus aureus* (MRSA). Int J Antimicrob Agents **30:**398–408.

[452] **Jones, R. N.** 2006. Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. Clin Infect Dis **42:**S13–24.

[453] **Soriano, A., F. Marco, J. A. Martínez, E. Pisos, M. Almela, V. P. Dimova, D. Alamo, M. Ortega, J. Lopez, and J. Mensa.** 2008. Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. Clin Infect Dis **46:**193–200.

[454] **Rybak, M. J., B. M. Lomaestro, J. C. Rotschafer, R. C. Moellering, W. A. Craig, M. Billeter, J. R. Dalovisio, and D. P. Levine.** 2009. Vancomycin therapeutic guidelines: a summary of consensus recommendations from the infectious diseases Society of America, the

American Society of Health-System Pharmacists, and the Society of Infectious Diseases Pharmacists. Clin Infect Dis **49:**325–7.

[455] **LaPlante, K. L., and M. J. Rybak.** 2004. Impact of high-inoculum *Staphylococcus aureus* on the activities of nafcillin, vancomycin, linezolid, and daptomycin, alone and in combination with gentamicin, in an *in vitro* pharmacodynamic model. Antimicrob Agents Chemother **48:**4665–72.

[456] **Sader, H. S., P. D. Fey, A. P. Limaye, N. Madinger, G. Pankey, J. Rahal, M. J. Rybak, D. R. Snydman, L. L. Steed, K. Waites, and R. N. Jones.** 2009. Evaluation of vancomycin and daptomycin potency trends (MIC creep) against methicillin-resistant *Staphylococcus aureus* isolates collected in nine U.S. medical centers from 2002 to 2006. Antimicrob Agents Chemother **53:**4127–32.

[457] **Traczewski, M. M., B. D. Katz, J. N. Steenbergen, and S. D. Brown.** 2009. Inhibitory and bactericidal activities of daptomycin, vancomycin, and teicoplanin against methicillin-resistant *Staphylococcus aureus* isolates collected from 1985 to 2007. Antimicrob Agents Chemother **53:**1735–8.

[458] **Appleman, M. D., and D. M. Citron.** 2010. Efficacy of vancomycin and daptomycin against *Staphylococcus aureus* isolates collected over 29 years. Diagn Microbiol Infect Dis **66:**441–4.

[459] **Hsu, D. I., L. K. Hidayat, R. Quist, J. Hindler, A. Karlsson, A. Yusof, and A. Wong-Beringer.** 2008. Comparison of method-specific vancomycin minimum inhibitory concentration values and their predictability for treatment outcome of meticillin-resistant *Staphylococcus aureus* (MRSA) infections. Int J Antimicrob Agents **32:**378–85.

[460] **Sakoulas, G., P. A. Moise-Broder, J. Schentag, A. Forrest, R. C. Moellering, and G. M. Eliopoulos.** 2004. Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. J Clin Microbiol **42:**2398–402.

[461] **Rojas, L., E. Bunsow, P. Muñoz, E. Cercenado, M. Rodríguez-Créixems, and E. Bouza.** 2012. Vancomycin MICs do not predict the outcome of methicillin-resistant *Staphylococcus aureus* bloodstream infections in correctly treated patients. J Antimicrob Chemother **67:**1760–8.

[462] **Sader, H. S., P. R. Rhomberg, and R. N. Jones.** 2009. Nine-hospital study comparing broth microdilution and Etest method results for vancomycin and daptomycin against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **53:**3162–5.

[463] **Howden, B. P., P. D. R. Johnson, P. B. Ward, T. P. Stinear, and J. K. Davies.** 2006. Isolates with low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. Antimicrob Agents Chemother **50:**3039–47.

[464] **Renzoni, A., P. Francois, D. Li, W. L. Kelley, D. P. Lew, P. Vaudaux, and J. Schrenzel.** 2004. Modulation of fibronectin adhesins and other virulence factors in a teicoplanin-resistant derivative of methicillin-resistant *Staphylococcus aureus.* Antimicrob Agents Chemother **48:**2958-65.

[465] **Sakoulas, G., G. M. Eliopoulos, R. C. Moellering, Jr., R. P. Novick, L. Venkataraman, C. Wennersten, P. C. DeGirolami, M. J. Schwaber, and H. S. Gold**. 2003. *Staphylococcus aureus* accessory gene regulator (*agr*) group II: is there a relationship to the development of intermediate-level glycopeptide resistance? J Infect Dis **187:**929–38.

[466] **Sakoulas, G., G. M. Eliopoulos, R. C. Moellering, C. Wennersten, L. Venkataraman, R. P. Novick, and H. S. Gold.** 2002. Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob Agents Chemother **46:**1492–502.

[467] **Tsuji, B. T., M. J. Rybak, C. M. Cheung, M. Amjad, and G. W. Kaatz.** 2007. Community- and health care-associated methicillin-resistant *Staphylococcus aureus*: a comparison of molecular epidemiology and

antimicrobial activities of various agents. Diagn Microbiol Infect Dis **58:**41–7.

[468] **Moise-Broder, P. A., G. Sakoulas, G. M. Eliopoulos, J. J. Schentag, A. Forrest, and R. C. Moellering. 2004.** Accessory gene regulator group II polymorphism in methicillin-resistant *Staphylococcus aureus* is predictive of failure of vancomycin therapy. Clin Infect Dis **38:**1700–5.

[469] **Verdier, I., M. E. Reverdy, J. Etienne, G. Lina, M. Bes, and F. Vandenesch.** 2004. *Staphylococcus aureus* isolates with reduced susceptibility to glycopeptides belong to accessory gene regulator group I or II. Antimicrob Agents Chemother **48:**1024–7.

[470] **Chong, Y. P., E. S. Kim, S. J. Park, K. H. Park, T. Kim, M. N. Kim, S. H. Kim, S. O. Lee, S. H. Choi, J. H. Woo, J. Y. Jeong, and Y. S. Kim.** 2013. Accessory gene regulator (*agr*) dysfunction in *Staphylococcus aureus* bloodstream isolates from South Korean patients. Antimicrob Agents Chemother **57:**1509–12.

[471] **Cui, L., T. Isii, M. Fukuda, T. Ochiai, H. M. Neoh, I. L. Camargo, Y. Watanabe, M. Shoji, T. Hishinuma, and K. Hiramatsu.** 2010. An RpoB mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus.* Antimicrob Agents Chemother **54:**5222–33.

[472] **Watanabe, Y., L. Cui, Y. Katayama, K. Kozue, and K. Hiramatsu.** 2011. Impact of *rpoB* mutations on reduced vancomycin susceptibility in *Staphylococcus aureus*. J Clin Microbiol **49:**2680–4.

[473] **Chen, C. J., M. H. Lin, J. C. Shu, and J. J. Lu.** 2014. Reduced susceptibility to vancomycin in isogenic *Staphylococcus aureus* strains of sequence type 59: Tracking evolution and identifying mutations by whole-genome sequencing. J Antimicrob Chemother **69:**349–54.

[474] **Satola, S. W., M. M. Farley, K. F. Anderson, and J. B. Patel.** 2011. Comparison of detection methods for heteroresistant vancomycin-intermediate *Staphylococcus aureus*, with the population analysis profile method as the reference method. J Clin Microbiol **49:**177–83.

[475] **Sánchez García, M., M. A. De la Torre, G. Morales, B. Peláez, M. J. Tolón, S. Domingo, F. J. Candel, R. Andrade, A. Arribi, N. García, F. Martínez Sagasti, J. Fereres, and J. Picazo.** Clinical outbreak of linezolid-resistant *Staphylococcus aureus* in an intensive care unit. JAMA **303:**2260–4.

[476] **Shore, A. C., O. M. Brennan, R. Ehricht, S. Monecke, S. Schwarz, P. Slickers, and D. C. Coleman.** 2010. Identification and characterization of the multidrug resistance gene cfr in a Panton-Valentine leukocidin-positive sequence type 8 methicillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. Antimicrob Agents Chemother **54:**4978–84.

[477] **Witte, W. and C. Cuny.** 2011. Emergence and spread of *cfr*-mediated multiresistance in staphylococci: an interdisciplinary challenge. Future Microbiol **6:**925–31.

[478] **Jones, R. N., J. E. Ross, M. Castanheira, and R. E. Mendes.** 2008. United States resistance surveillance results for linezolid (LEADER Program for 2007). Diagn Microbiol Infect Dis **62:**416–26.

[479] **Pérez-Vázquez, M., A. Vindel, C. Marcos, J. Oteo, O. Cuevas, P. Trincado, V. Bautista, H. Grundmann, and J. Campos; EARSS Spain *spa*-typing Group.** 2009. 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 **63:**21–31.

[480] **Oliveira, D. C., A. Tomasz, and H. De Lencastre.** 2002. Secrets of success of a human pathogen: Molecular evolution of pandemic clones of meticillin-resistant *Staphylococcus aureus.* Lancet Infect Dis 2:180–9.

[481] **Espadinha, D., N. A.. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, and H. de Lencastre; Médicos Sentinela Network.** 2013. Extensive dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) between the hospital and the community in a country with a high prevalence of nosocomial MRSA. PLoS One **8:**e59960.

[482] **Argudín, M. A., M. C. Mendoza, F. J. Méndez, M. C. Martín, B. Guerra, and M. R. Rodicio.** 2009. 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 **47:** 2097–105.

[483] **Torres-Sangiao, E., S. Pérez-Castro, M. I. Fernández-Natal, R. Cisterna-Cáncer, M. Zapico-González, B. Fernández-Pérez, E. Ojeda-Fernández, T. Nebreda, M. Gozalo-Margüello, C. Fuster-Foz, M. P. Roiz-Mesones, M. D. Miguel-Martín, L. Torroba, A. Coira-Nieto, F. Vasallo-Vidal, S. Méndez-Lage, E. Prieto-Rodriguez, J. M. Eiros, J. Torres, and G. Bou.** 2012. Identification of international circulating lineages of meticillin-resistant *Staphylococcus aureus* in the north of Spain and their glycopeptide and linezolid susceptibility. J *Med Microbiol* **61:**305–7.

[484] **Pérez-Roth, E., F. Lorenzo-Díaz, N. Batista, A. Moreno, and S. Méndez-Álvarez.** 2004. Tracking methicillin-resistant *Staphylococcus aureus* clones during a 5-year period (1998 to 2002) in a Spanish hospital. J Clin Microbiol **42:**4649–56.

[485] **Grundmann, H., L. M. Schouls, D. M. Aanensen, G. N. Pluister, A. Tami, M. Chlebowicz, C. Glasner, A. J. Sabat, K. Weist, O. Heuer, and A. W. Friedrich; ESCMID Study group on Molecular Epidemiological Markers; European Staphylococcal Reference Laboratory Working Group.** 2014. The dynamic changes of dominant clones of *Staphylococcus aureus* causing bloodstream infections in the European region : results of a second structured survey. Eurosurveillance **19:**pii:20987.

[486] **Chambers, H. F., and F. R. Deleo.** 2010. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol **7:**629–41.

[487] **Stefani, S., D. R. Chung, J. A. Lindsay, A. W. Friedrich, A. M. Kearns, H. Westh, and F. M. Mackenzie.** 2012. Meticillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation

of typing methods. Int J Antimicrob Agents **39:**273–82.

[488] **Campanile, F., D. Bongiorno, M. Falcone, F. Vailati, M. B. Pasticci, M. Perez, A. Raglio, F. Rumpianesi, C. Scuderi, F. Suter, M. Venditti, C. Venturelli, V. Ravasio, M. Codeluppi, and S. Stefani.** 2012. Changing Italian nosocomial-community trends and heteroresistance in *Staphylococcus aureus* from bacteremia and endocarditis. Eur J Clin Microbiol Infect Dis **31:**739–45.

[489] **Oteo, J., F. Baquero, A. Vindel, and J. Campos; Spanish members of the European Antimicrobial Resistance Surveillance System.** 2004. Antibiotic resistance in 3113 blood isolates of *Staphylococcus aureus* in 40 Spanish hospitals participating in the European Antimicrobial Resistance Surveillance System (2000-2002). J Antimicrob Chemother **53:**1033–8.

[490] **Johnson, A. P., H. M. Aucken, S. Cavendish, M. Ganner, M. C. Wale, M. Warner, D. M. Livermore, and B. D. Cookson; UK EARSS participants.** 2001. Dominance of EMRSA-15 and -16 among MRSA causing nosocomial bacteraemia in the UK: analysis of isolates from the European Antimicrobial Resistance Surveillance System (EARSS). J Antimicrob Chemother **48:**143–4.

[491] **Fossum Moen, A. E., J. Saltyte Benth, K. Alm-Kristiansen, and G. Bukholm.** 2009. Exotoxin-encoding gene content in community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect **15:**1139–45.

[492] **Melter, O., P. Urbásková, V. Jakubů, B. Macková, and H. Zemlicková; Czech participants in EARSS.** Emergence of EMRSA-15 clone in hospitals throughout the Czech Republic. Euro Surveill **11:**E060803.6.

[493] **Ellington, M. J., M. Ganner, M. Warner, E. Boakes, B. D. Cookson, R. L. Hill, and A. M. Kearns.** 2010. First international spread and dissemination of the virulent Queensland community-associated methicillin-resistant *Staphylococcus aureus* strain. Clin Microbiol Infect **16:**1009–12.

[494] **Yamamoto, T., W. C. Hung, T. Takano, and A. Nishiyama.** 2013. Genetic nature and virulence of community-associated methicillin-resistant *Staphylococcus aureus.* BioMedicine **3:**2–18.

[495] **Wisplinghoff, H., B. Ewertz, S. Wisplinghoff, D. Stefanik, G. Plum, F. Perdreau-Remington, and H. Seifert**. 2005. Molecular evolution of methicillin-resistant *Staphylococcus aureus* in the metropolitan area of Cologne, Germany, from 1984 to 1998. J Clin Microbiol **43:**5445–51.

[496] **Blanco, R., A. Tristan, G. Ezpeleta, A. R. Larsen, M. Bes, J. Etienne, R. Cisterna, and F. Laurent.** 2011. Molecular epidemiology of Panton-Valentine leukocidin-positive *Staphylococcus aureus* in Spain: emergence of the USA300 clone in an autochthonous population. J Clin Microbiol **49:**433–6.

[497] **Fernandez, S., S. Murzicato, O. Sandoval, L. Fernández-Canigia, and M. Mollerach.** 2015. Community-acquired necrotizing pneumonia caused by methicillin-resistant *Staphylococcus aureus* ST30-SCC*mec*IVc-spat019-PVL positive in San Antonio de Areco, Argentina. Rev Argent Microbiol **47:**50–3.

[498] **Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich; European Staphylococcal Reference Laboratory Working Group.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med **7:**e1000215.

[499] **Vainio, A., S. Koskela, A. Virolainen, J. Vuopio, and S. Salmenlinna.** 2011. “Adapting *spa* typing for national laboratory-based surveillance of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis **30:**789–97.

[500] **Coombs, G. W., S. Monecke, J. C. Pearson, H. L. Tan, Y. K. Chew, L. Wilson, R. Ehricht, F. G. O’Brien, and K. J. Christiansen.** 2011. Evolution and diversity of community-associated methicillin-resistant *Staphylococcus aureus* in a geographical region. BMC Microbiol **11:**215.

[501] **Jiménez, J. N., A. M. Ocampo, J. M. Vanegas, E. A. Rodriguez, J. R. Mediavilla, L. Chen, C. E. Muskus, L. A. Vélez, C. Rojas, A. V Restrepo, S. Ospina, C. Garcés, L. Franco, P. Bifani, B. N. Kreiswirth, and M. M. Correa.** 2012. CC8 MRSA strains harboring SCC*mec* type IVc are predominant in Colombian hospitals. PLoS One **7:**e38576.

[502] **Denis, O., C. Suetens, M. Hallin, B. Catry, I. Ramboer, M. Dispas, G. Willems, B. Gordts, P. Butaye, and M. J. Struelens**. 2009. Methicillin-resistant *Staphylococcus aureus* ST398 in swine farm personnel, Belgium. Emerg Infect Dis **15:**1098–101.

[503] **Cuny, C., R. Nathaus, F. Layer, B. Strommenger, D. Altmann, and W. Witte.** 2009. Nasal colonization of humans with methicillin-resistant *Staphylococcus aureus* (MRSA) CC398 with and without exposure to pigs. PLoS One. **4:**e6800.

[504] **Kadlec, K., A. T. Feßler, T. Hauschild, and S. Schwarz.** 2012. Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus.* Clin Microbiol Infect **18:**745–55.

[505] **Lozano, C., C. Aspiroz, Y. Sáenz, M. Ruiz-García, G. Royo-García, E. Gómez-Sanz, F. Ruiz-Larrea, M. Zarazaga, and C. Torres.** 2012. Genetic environment and location of the *lnu*(A) and *lnu*(B) genes in methicillin-resistant *Staphylococcus aureus* and other staphylococci of animal and human origin. J Antimicrob Chemother **67:**2804–8.

[506] **Lo, Y. P., M. T. Wan, M. M. Chen, H. Y. Su, T. L. Lauderdale, and C. C. Chou.** 2012. Molecular characterization and clonal genetic diversity of methicillin-resistant *Staphylococcus aureus* of pig origin in Taiwan. Comp Immunol Microbiol Infect Dis **35:**513–21.

[507] **Wellinder-Olsson, C., K. Florén-Johansson, L. Larsson, S. Oberg, L. Karlsson, and C. Ahrén.** 2008. Infection with Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* t034.," Clin Infect Dis **14:**1271–2.

[508] **Schaumburg, F., R. Köck, A. Mellmann, L. Richter, F. Hasenberg, A. Kriegeskorte, A. W. Friedrich, S. Gatermann, G. Peters, C. von Eiff, and K. Becker.** 2012. Population dynamics among methicillin resistant *Staphylococcus aureus* in Germany during a 6-year period. J Clin Microbiol **50:**3186–92.

[509] **Camoez, M., J. M. Sierra, M. Pujol, A. Hornero, R. Martin, and M. A. Domínguez**. 2013. Prevalence and molecular characterization of methicillin-resistant *Staphylococcus aureus* ST398 resistant to tetracycline at a Spanish Hospital over 12 Years. PLoS One **8:**e72828.

[510] **European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption**. 2013. Sales of veterinary antimicrobial agents in 25 EU/EEA countries in 2011 (EMA/236501/2013).

[511] **Stegger, M., C. M. Liu, J. Larsen, K. Soldanova, M. Aziz, T. Contente-Cuomo, A. Petersen, S. Vandendriessche, J. N. Jiménez, C. Mammina, A. van Belkum, S. Salmenlinna, F. Laurent, R. L. Skov, A. R. Larsen, P. S. Andersen, and L. B. Price.** 2013. Rapid differentiation between livestock-associated and livestock-independent *Staphylococcus aureus* CC398 clades. PLoS One **8:**e79645.

[512] **Uhlemann, A. C., S. F. Porcella, S. Trivedi, S. B. Sullivan, C. Hafer, A. D. Kennedy, K. D. Barbian, A. J. Mccarthy, C. Street, D. L. Hirschberg, W. I. Lipkin, J. A.. Lindsay, F. R. Deleo, and F. D. Lowy.** 2012. Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with distinct genomic and cell adhesion properties. MBio **3:**piie00027-12.

[513] **McCarthy, A. J., A. A. Witney, K. A. Gould, A. Moodley, L. Guardabassi, A. Voss, O. Denis, E. M. Broens, J. Hinds, and J. A. Lindsay.** 2011. The distribution of mobile genetic elements (MGEs) in MRSA CC398 Is associated with both host and country. Genome Biol Evol **3:**1164–74.

[514] **Huijsdens, X. W., T. Bosch, M. G. van Santen-Verheuvel, E. Spalburg, G. N. Pluister, M. van Luit, M. E. Heck, A. Haenen, and A. J. de**

Neeling. 2009. Molecular characterization of PFGE non-typable methicillin-resistant *Staphylococcus aureus* in The Netherlands, 2007. Euro Surveill **14:**pii:19335.

[515] **Williamson, D. A., S. Bakker, G. W. Coombs, H. Leen Tan, S. Monecke, and H. Heffernan.** 2014. Emergence and molecular characterization of clonal complex 398 (CC398) methicillin-resistant *Staphylococcus aureus* (MRSA) in New Zealand. J Antimicrob Chemother **69:**1428–30.

[516] **Fluit, A. C.** 2012. Livestock-associated *Staphylococcus aureus*. Clin Microbiol Infect **18:**735–44.

[517] **Kuhn, G., T. Koessler, D. C. Melles, P. Francois, A. Huyghe, P. Dunman, M. C. Vos, G. Zanetti, J. Schrenzel, A. van Belkum, and D. S. Blanc.** 2010. Comparative genomics of epidemic versus sporadic *Staphylococcus aureus* strains does not reveal molecular markers for epidemicity. Infect Genet Evol **10:**89–96.

[518] **Gherardi, G., L. De Florio, G. Lorino, L. Fico, and G. Dicuonzo.** 2009. Macrolide resistance genotypes and phenotypes among erythromycin-resistant clinical isolates of *Staphylococcus aureus* and coagulase-negative staphylococci, Italy. FEMS Immunol Med Microbiol **55:**62–7.

[519] **Shore, A. C., O. M. Brennan, E. C. Deasy, A. S. Rossney, P. M. Kinnevey, R. Ehricht, S. Monecke, and D. C. Coleman.** 2012. DNA microarray profiling of a diverse collection of nosocomial methicillin-resistant *Staphylococcus aureus* isolates assigns the majority to the correct sequence type and staphylococcal cassette chromosome *mec* (SCC*mec*) type and results in the subsequent identification and characterization of novel SCC*mec*-SCCM1 composite islands. Antimicrob Agents Chemother **56:**5340–55.

[520] **Schmitz, F. J., A. Krey, R. Sadurski, J. Verhoef, D. Milatovic, and A. C. Fluit; European SENTRY Participants.** 2001. Resistance to tetracycline and distribution of tetracycline resistance genes in European *Staphylococcus aureus* isolates. J Antimicrob Chemother **47:**239–40.

[521] **Shittu, A., O. Oyedara, F. Abegunrin, K. Okon, A. Raji, S. Taiwo, F. Ogunsola, K. Onyedibe, and G. Elisha.** 2012. Characterization of methicillin-susceptible and -resistant staphylococci in the clinical setting: a multicentre study in Nigeria. BMC Infect Dis **12:**286.

[522] **DiCicco, M., S. Weese, S. Neethirajan, J. Rousseau, and A. Singh.** 2014. Fosfomycin susceptibility of canine methicillin-resistant *Staphylococcus pseudintermedius* isolates. Res Vet Sci **96:**251–3.

[523] **Wolter, D. J., A. Chatterjee, M. Varman, and R. V Goering**. 2008. Isolation and characterization of an epidemic methicillin-resistant *Staphylococcus aureus* 15 variant in the central United States. J Clin Microbiol **46:**3548–9.

[524] **Geraci, D. M., M. Giuffrè, C. Bonura, D. Matranga, A. Aleo, L. Saporito, G. Corsello, A. R. Larsen, and C. Mammina.** 2014. Methicillin-resistant *Staphylococcus aureus* colonization: a three-year prospective study in a neonatal intensive care unit in Italy. PLoS One **9:**e87760.

[525] **Al Laham, N., J. R. Mediavilla, L. Chen, N. Abdelateef, F. A. Elamreen, C. C. Ginocchio, D. Pierard, K. Becker, and B. N. Kreiswirth.** 2015. MRSA clonal complex 22 strains harboring toxic shock syndrome toxin (TSST-1) are endemic in the primary hospital in Gaza, Palestine. PLoS One **10:**e0120008.

[526] **Bukowski, M., B. Wladyka, and G. Dubin.** 2010. Exfoliative toxins of *Staphylococcus aureus*. Toxins (Basel) **2:**1148–65.

[527] **Koosha, R. Z., A. A. Fooladi, H. M. Hosseini, and E. M. Aghdam.** 2014. Prevalence of exfoliative toxin A and B genes in *Staphylococcus aureus* isolated from clinical specimens. J Infect Public Health **7:**177–85.

[528] **Melter, O., M. Aires de Sousa, P. Urbásková, V. Jakubů, H. Zemlicková, and H. de Lencastre.** 2003. Update on the major clonal types of methicillin-resistant *Staphylococcus aureus* in the Czech Republic. J Clin Microbiol **41:**4998–5005.

[529] **Smyth, D. S., W. J. Meaney, P. J. Hartigan, and C. J. Smyth.** 2007. Occurrence of *ssl* genes in isolates of *Staphylococcus aureus* from animal infection. J Med Microbiol **56:**418–25.

[530] **McNicholas, S., A. C. Shore, D. C. Coleman, H. Humphreys, and D. F. Hughes.** 2011. DNA microarray genotyping and virulence and antimicrobial resistance gene profiling of methicillin-resistant *Staphylococcus aureus* bloodstream isolates from renal patients. J Clin Microbiol **49:**4349–51.

Annex I

Thesis articles

Article 1: Sierra JM, **Camoez M,** Tubau F, Gasch O, Pujol M, Martin R, Domínguez MA. Low Prevalence of *cfr*-Mediated linezolid resistance among methicillin-resistant *Staphylococcus aureus* in a Spanish hospital: case report on linezolid resistance acquired during linezolid therapy. *PLoSOne*. 2013;8(3):e59215. **(IF: 3.234)**

Article 2: **Camoez M,** Sierra JM, Pujol M, Hornero A, Martin R, Domínguez MA. Prevalence and molecular characterization of methicillin-resistant *Staphylococcus aureus* ST398 resistant to tetracycline at a Spanish hospital over 12 years. *PLosOne*. 2013;8(9):e72828. **(IF: 3.234)**

Article 3: **Camoez M,** Càmara J, Tubau F, Hornero A, Pujol M, Liñares J, Domínguez MA. Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Barcelona, Spain, 2012-2013. *Eur J Clin Microbiol Infect Dis* (In submission process to the European Journal of Clinical Microbiology & Infectious Diseases)

Article 4: **Camoez M,** Sierra JM, Dominguez MA, Ferrer-Navarro M, Vila J, Roca I. Automated categorization of methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin Microbiol Infect*. 2015 Oct 16. pii: S1198-743X(15)00908-8. doi: 10.1016/j.cmi.2015.10.009. **(IF: 5.768)**

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Low Prevalence of C*fr*-Mediated Linezolid Resistance among Methicillin-Resistant *Staphylococcus aureus* in a Spanish Hospital: Case Report on Linezolid Resistance Acquired during Linezolid Therapy

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Abstract

Linezolid is an effective antimicrobial agent to treat methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance to linezolid due to the *cfr* gene is described worldwide. The present study aimed to analyze the prevalence of the *cfr*–mediated linezolid resistance among MRSA clinical isolates in our area. A very low prevalence of *cfr* mediated linezolid resistance was found: only one bacteremic isolate out of 2 215 screened isolates. The only linezolid resistant isolate arose in a patient, previously colonized by MRSA, following linezolid therapy. Despite the low rate of resistance in our area, ongoing surveillance is advisable to avoid the spread of linezolid resistance.

Citation: Sierra JM, Camoez M, Tubau F, Gasch O, Pujol M, et al. (2013) Low Prevalence of C*fr*-Mediated Linezolid Resistance among Methicillin-Resistant *Staphylococcus aureus* in a Spanish Hospital: Case Report on Linezolid Resistance Acquired during Linezolid Therapy. PLoS ONE 8(3): e59215. doi:10.1371/journal.pone.0059215

Editor: Holger Rohde, Universitätsklinikum Hamburg-Eppendorf, Germany

Received November 13, 2012; **Accepted** February 12, 2013; **Published** March 15, 2013

Funding: This work was supported by a grant from Fondo de Investigación Sanitaria (grant number 10/01783) and by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD06/0008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Linezolid has been introduced into the medical practice to treat Gram-positive infections, especially those related with staphylococcal infections including methicillin resistance *Staphylococcus aureus* (MRSA).

Since the introduction of linezolid in the clinical practice several mechanisms of linezolid-resistance have been described. The major mechanism of resistance is mediated by mutations in the V domain of the 23S rRNA. The most common mutation found is located in the position 2,576 (*E.coli* numbering). Other mutations close to the 2,576 position could also confer linezolid resistance [1,2]. Due to the presence of multiple copies of the 23SrRNA gene, a relationship between the number of mutated genes and the level of resistance is well established, and known as "gene dosage" [3]. Another mechanism involved in linezolid resistance is the modification of ribosomal proteins L3 and L4 encoded by *rplC* and *rplD* genes, respectively. Some of the mutations found in these genes are concurrent with mutations in the V domain of the 23S rRNA [4]. Finally, RNA methylation by two different methyltranferases has been related to linezolid resistance: *RlnM* - a codon insertion in the methyltransferase gene *rlmN* reduces linezolid susceptibility in clinical *S.aureus* [5], and a methyltranferase encoded by the *cfr* gene. The *cfr* gene is mostly plasmid-located [6] and confers cross resistance to phenicols, lincosamides, oxazolidinones, pleromutilines amd streptogramin A (PhLOPS phenotype). Some outbreaks of *crf*-mediated linezolid-resistant strains have been reported in the literature, such as the recent outbreak described in Spain by Morales et al [7]. In this case, the epidemic strain was involved in surgical site infections; ventilator-assisted pneumonia; and primary bacteremia in an intensive care unit, affecting a total of 12 patients.

The objective of our work was to evaluate the prevalence of *cfr* gene among MRSA clinical isolates in Hospital Universitari de Bellvitge (HUB) from 1999 to 2010.

Materials and Methods

In the HUB 2,215 clinical MRSA isolates from single patients were isolated from 1999 to 2010. Antibiotic susceptibility to cefoxitin, oxacillin, erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, tetracycline, rifampin, chloramphenicol, vancomycin and teicoplanin was studied in all strains by the disc diffusion method, following CLSI guidelines. Isolates resistant to both clindamycin and chloramphenicol, potentially linezolid-resistant, were screened for the *cfr* presence. Susceptibility to linezolid was studied in this group by the disc diffusion method and microdilution (0.06 mg/L to 128 mg/L) according to CLSI guidelines. The presence of *cfr* gene was analyzed by PCR. Two strains carrying the *cfr* gene and previously characterized [8] were

Table 1. Linezolid susceptibility by MIC and disc-diffusion in 16 clindamycin and chloramphenicol resistant MRSA isolates and presence of the *cfr* gene.

Number of strains	Linezolid MIC (mg/L)	Linezolid inhibition zone (mm)	*cfr* gene
15	1–2	29–32	−
1	8	25	+
Control strains (n = 2)	8–16	26	+

doi:10.1371/journal.pone.0059215.t001

used as controls. Linezolid-resistant strains were genotyped by multilocus sequence typing (MLST), following the methodology described by Enrigh *et al.* [9], sequence types (STs) were determined by comparing with the MLST database (www.mlst.net). Staphylococcal Chromosome Cassette *mec* (SCC*mec*) typing and *agr* polymorphism were studied by PCR according to previously published procedures [10,11].

Results and Discussion

Linezolid was introduced in the clinical practice in HUB during 2003. Linezolid prescription average in our hospital during the 2004–2010 period was of 0.28 defined daily dose (DDD)/100 patients-days, with a peak of 0.50 DDD/100 patients-days in 2009.

Only 16 MRSA isolates (0.7%) had the clindamycin and chloramphenicol resistance profile. Linezolid MICs were ≤2 mg/L in all but one strain which showed MIC of 8 mg/L and carried the *cfr* gene. Summarized MICs and results are shown in table 1. The linezolid resistant strain was isolated from a blood culture in April 2009 from a 76 year-old man. The patient had been admitted to the intensive care unit (ICU) in February 2009 because of complications derived from a laryngeal cancer surgery performed on December 2008. The patient carried a nasal MRSA on admittance. During March multiple antibiotics were administered, including linezolid over 19 days, to treat a MRSA respiratory low-tract infection. The initial respiratory MRSA isolates as well as the nasal isolate were resistant to erithromycin, clindamycin, gentamycin, tobramycin, ciprofloxacin, rifampin and susceptible to chloramphenicol and linezolid. The linezolid-resistant MRSA strain was isolated, after linezolid administration, in a single central catheter blood culture and subsequently in different respiratory samples, though no specific therapy was adopted. The patient finally died on May 2009, from a cardiac arrest of unknown etiology. The linezolid resistant isolate was also resistant to erithromycin, clindamycin, gentamycin, tobramycin, ciprofloxacin, rifampin and chloramphenicol and belonged to clone ST228-MRSA-SCC*mec*I with *agr* type II. Linezolid susceptible MRSA strains also belonged to clone ST228-MRSA-SCC*mec*I. No further spread of the linezolid resistant strain to other patients was detected.

Other reports worldwide described the presence of the *cfr* gene in different *S.aureus* genotypes either of community, ST8-MRSA-SCC*mec*IV and ST398-MRSA-SCC*mec*V [12,13], or nosocomial origin, ST125-MRSA-SCC*mec*IVc [8]. Thus, the presence of this mechanism of resistance in different *S.aureus* genotypes could potentially be easily spread worldwide due to its plasmid location. In the case reported here, we did not perform additional studies on coagulase-negative *staphylococci*, isolated from the same patient or from other patients admitted to the ICU, that could play a role as potential reservoir of the *cfr* gene for MRSA strains.

In our series, overall linezolid resistance mediated by the *cfr* gene is very low in this period (0.05%; 1/2,215), similar to other surveillance studies [14]. Among clindamycin and chloramphenicol resistant strains, *cfr*-mediated linezolid resistance was 6.25% (1/16). Kerenberg et al [15] found the *cfr* gene in 3% of chloramphenicol-resistant strains of *Staphylococcus spp.* of animal and human origin.

The disc-diffusion technique is not suitable to recognize linezolid resistance mediated by the *cfr* gene with the current CLSI or EUCAST breakpoints. In our experience, staphylococcal isolates exhibiting a resistance phenotype to clindamycin, chloramphenicol and linezolid could suggest for a possible presence of *cfr*. However, further molecular investigations are needed due to the low *cfr* prevalence (6.25%) observed in this studied population. Other linezolid resistance mechanisms, non *cfr*-mediated, are possible, but they do not necessarily involve clindamycin and chloramphenicol resistance.

Acknowledgments

We thank Dr Ruiz de Gopegui, from Servicio de Microbiología, Hospital Universitari Son Espases, Palma de Mallorca, Spain, for sending us *cfr*-control strains.

Author Contributions

Conceived and designed the experiments: JMS MC FT OG MP MAD. Performed the experiments: JMS MC MAD. Analyzed the data: JMS MC FT OG MP RM MAD. Contributed reagents/materials/analysis tools: JMS MC MAD. Wrote the paper: JMS MC RM MAD.

References

1. Bozdogan B, Appelbaum PC (2004) Oxazolidinones: activity, mode of action, and mechanism of resistance. Int J Antimicrob Agents 23: 113–119.
2. Livermore DM (2003) Linezolid *in vitro*: mechanism and antibacterial spectrum. J Antimicrob Chemother 51: Suppl 2 ii 9–16.
3. Besier S, Ludwig A, Zander J, Brade V, Wichelhaus TA (2008) Linezolid resistance in *Staphylococcus aureus*: gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob Agents Chemother 52: 1570–1572.
4. Locke JB, Hilgers M, Shaw KJ (2009) Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in staphylococci of clinical origin. Antimicrob Agents Chemother 53: 5275–5278.
5. Gao W, Chua K, Davies JK, Newton HJ, Seemann T, et al. (2010) Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog 6: e1000944.
6. Arias CA, Vallejo M, Reyes J, Panesso D, Moreno J, et al. (2008) Clinical and microbiological aspects of linezolid resistance mediated by the *cfr* gene encoding a 23S rRNA methyltransferase. J Clin Microbiol 46: 892–896.
7. Morales G, Picazo JJ, Baos E, Candel FJ, Arribi A, et al. (2010) Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. Clin Infect Dis 50: 821–825.
8. Ruiz de Gopegui E, Juan C, Zamorano L, Perez JL, Oliver A (2012) Transferable multidrug resistance plasmid carrying *cfr* associated with *tet*(L), *ant*(4')-Ia and *dfr*K genes from a clinical methicillin-resistant *Staphylococcus aureus* ST125 strain. Antimicrob Agents Chemother 56: 2139–2142.

9. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol 38: 1008–1015.
10. Milheirico C, Oliveira DC, de Lencastre H (2007) Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob Agents Chemother 51: 3374–3377.
11. Lina G, Boutite F, Tristan A, Bes M, Etienne J, et al. (2003) Bacterial competition for human nasal cavity colonization: role of Staphylococcal *agr* alleles. Appl Environ Microbiol 69: 18–23.
12. Shore AC, Brennan OM, Ehricht R, Monecke S, Schwarz S, et al (2010) Identification and characterization of the multidrug resistance gene *cfr* in a Panton-Valentine leukocidin-positive sequence type 8 methicillin-resistant *Staphylococcus aureus* IVa (USA300) isolate. Antimicrob Agents Chemother 54: 4978–4984.
13. Witte W, Cuny C (2011) Emergence and spread of *cfr*-mediated multiresistance in staphylococci: an interdisciplinary challenge. Future Microbiol 6: 925–931.
14. Jones RN, Ross JE, Castanheira M, Mendes RE (2008) United States resistance surveillance results for linezolid (LEADER Program for 2007). Diagn Microbiol Infect Dis 62: 416–426.
15. Kehrenberg C, Schwarz S (2006) Distribution of florfenicol resistance genes *fex*A and cfr among chloramphenicol-resistant *Staphylococcus isolates*. Antimicrob Agents Chemother 50: 1156–1163.

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Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* ST398 Resistant to Tetracycline at a Spanish Hospital over 12 Years

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398, associated with livestock animals, was described in 2003 as a new lineage infecting or colonizing humans. We evaluated the prevalence and molecular characteristics of MRSA ST398 isolated in the Hospital Universitari de Bellvitge from January 2000 to June 2011. Tetracycline resistant (Tet-R) MRSA isolates from single patients (pts) were screened by *Sma*I-pulsed field gel electrophoresis (PFGE). Nontypable MRSA strains by *Sma*I (NT_{SmaI})-MRSA were further analysed by *Apa*I-PFGE, *spa*, SCC*mec, agr*, MLST typing, and by DNA microarray hybridization. Among 164 pts harboring Tet-R MRSA, NT_{SmaI}-MRSA ST398-*agr*I was found in 33 pts (20%). Although the first pt was detected in 2003, 22/33 pts (67%) were registered in the 2010–2011 period. Ten pts (30%) were infected and cancer was the most frequent underlying disease. In one case, death was due to MRSA-ST398-related infection. Five pulsotypes (A–E) were detected using *Apa*I-PFGE, with type A accounting for 76% of the strains. The majority of the studied isolates presented *spa* type t011 (70%) and SCC*mec* type V (88%). One strain was *spa* negative both by PCR and microarray analysis. Forty-nine percent of the studied isolates showed resistance to 3 or more antibiotic classes, in addition to beta-lactams. Ciprofloxacin resistance was 67%. Tet-R was mediated by *tet*(M) and *tet*(K) in 26 isolates. All isolates lacked Panton-Valentine Leukocidin production, as well as other significant toxins. This study displays the molecular features of MRSA-ST398 clone and shows the increase in tetracycline resistance together with arise in MRSA-ST398 isolates infecting or colonizing patients in our clinical setting.

Citation: Camoez M, Sierra JM, Pujol M, Hornero A, Martin R, et al. (2013) Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* ST398 Resistant to Tetracycline at a Spanish Hospital over 12 Years. PLoS ONE 8(9): e72828. doi:10.1371/journal.pone.0072828

Editor: Holger Rohde, Universitätsklinikum Hamburg-Eppendorf, Germany

Received February 12, 2013; **Accepted** July 14, 2013; **Published** September 5, 2013

Funding: This work was supported by a grant from Fondo de Investigación Sanitaria (grant number 10/01783) and by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD06/0008).The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen causing nosocomial and community-acquired infections worldwide [1]. In recent years, community acquired MRSA strains that are genetically unrelated to the traditional hospital have emerged [2]. MRSA Clonal Complex (CC) 398 associated with livestock (LA-MRSA) has been described as a new clonal lineage infecting or colonizing humans in several countries around the world [3].

According to several studies, human's exposure to livestock constitutes a risk-factor for carriage of MRSA CC398 strains an' development of a possible infection [4]. Carriage prevalence in livestock farming profession is very high [1,5], but some strains have been detected in people without risk factors [6]. The majority of the MRSA sequence type (ST) 398 strains are related to skin and soft tissue infections (SSTIs) [7]. However severe infections can occur and have been reported in Europe, Asia, and the United States [8,9,10,11]. Although there is little information on the percentage of this clone associated to human infections, the Netherlands, Belgium, Denmark, Germany and Austria appear to be the countries that have more cases [8]. MRSA-ST398 was first reported in Spain in 2010 associated to a skin lesion [12].

Some microbiologic traits define isolates belonging to the MRSA-ST398 clone: chromosomal DNA cannot be restricted by *Sma*I enzyme and CC398 strains generally present resistance to tetracycline, which is commonly used in pig farming [1,13,14]. The absence of certain important virulence factors such as Panton-Valentine Leukocidin (PVL) and Toxic Shock Syndrome Toxin (TSST) seems to be common in CC398 isolate. Nevertheless, this clone has been associated with both animal and human disease [14].

The aims of this study were to evaluate the prevalence and molecular characteristics of MRSA ST398, isolated in the Hospital Universitari de Bellvitge from January 2000 to June 2011.

Materials and Methods

Clinical Setting

The present study has been approved by the Clinical Research Ethics Committee of the Hospital Universitari de Bellvitge. The Ethics Committee granted an exemption on obtaining the patients informed consent as this retrospective study focused on bacteria characterization and did not require any specific patient involvement. The study was conducted at the Hospital Universitari de Bellvitge (HUB), a 900-bed tertiary care academic institution located in the Barcelona metropolitan area, Spain. It is a reference centre for adult patients with approximately 35,000 admissions per year providing medical and surgical care for a population of 1,000,000 inhabitants. Most of the patients live and work in urban areas; however, the HUB is also the reference hospital for some rural population living to the south of the city of Barcelona. All episodes of infection or colonization by MRSA of ST398 were reviewed. Clinical information including patient age, sex, associated diseases and infection source were recorded.

Bacterial Strains

S. aureus isolates were identified using standard tests: catalase, latex agglutination (Microgen Staph, Microgen Biproducts, Camberley, England) and tube coagulase test (Staph-ase, bioMérieux, Marcy l-Étoile, France).

The average rate of total MRSA among *S. aureus* clinical isolates in our hospital was 21% for the 2000–2011 period (ranging from 15% in 2001 to 26% in 2006). A total of 184 MRSA tetracycline resistant (Tet-R) strains were isolated from single patients from January 2000 to December 2011, accounting for 6% of all MRSA recovered during this time period. One hundred and sixty-four of these Tet-R MRSA strains were available for this study. We failed to re-culture the remaining 20 isolates so they could not be included in the study.

Susceptibility Testing

Susceptibility testing was carried out by the disk-diffusion method following the Clinical and Laboratory Standards Institute (CLSI) recommendations [14,15]. The antibiotics tested were: penicillin (10 units), oxacillin (1 μg), cefoxitin (30 μg), erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), tobramycin (10 μg), rifampicin (5 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), teicoplanin (30 μg), quinupristin/dalfopristin (15 μg), and linezolid (30 μg). Vancomycin and daptomycin susceptibility was studied by broth microdilution.

Molecular Typing and DNA microarray hybridization

Pulsed Field Gel Electrophoresis (PFGE). The Tet-R MRSA isolates available for the study (n = 164) were tested for *Sma*I restriction as previously described [16]. Nontypable MRSA strains by *Sma*I (NT_{SmaI})-MRSA were further re-analyzed by *Apa*I-PFGE with pulse times from 0.5 to 15 s for 20 h. The resulting restriction patterns by *Apa*I enzyme, were interpreted both by visual inspection, using the criteria of van Belkum *et al.* [17], and by analysis with the FINGERPRINTING TM II software, version 3.0 (BioRad Laboratories, Inc., Madrid, Spain). The dendogram was generated using the unweighted-pair group with arithmetic averages method based on Dice coefficients, where optimization and band position tolerance were both set at 0.7%. PFGE type clusters were defined by a similarity coefficient of 80%.

Multilocus sequence typing (MLST). MLST was performed on representative strains of each *Apa*I-PFGE subtype as described by Enright *et al* [18]. *S. aureus* MLST database (http://www.mlst.net) was used to assign sequence types.

agr typing. The detection of *agr* polymorphism was performed in all strains using the multiplex PCR as described previously [19].

SCCmec typing. The staphylococcal cassette chromosome *mec* (SCC*mec*) type was characterized for all the NT_{SmaI}-MRSA isolates, using the multiplex PCR strategy developed by Milheiriço *et al.* [20].

spa typing. All NT_{SmaI}-MRSA strains were characterized by *spa* typing as described previously [21] using the Ridom StaphType software, version 1.4 (Ridom GmbH Münster, Germany) and the *spa* types were assigned according to the Ridom web server (http://www.spaserver.ridom.de). The BURP algorithm was used to calculate *spa* clonal complexes (*spa*CC) with the defaults parameters set by the software, i.e. *spa* types shorter than five repeats were excluded and *spa* types were clustered in the same *spa*CC if cost was less or equal to six.

DNA microarray hybridization. DNA microarray hybridization (Staphy-Type Kit, Alere Technologies Ltd, Jena, Germany; stripe version) was conducted in all NT_{SmaI}-MRSA strains following the manufacturer's guidelines. The protocols and procedures have been previously described in detail [22]. Briefly, this microarray contains 334 probes, including approximately 180 different genes and their allelic variants. Target genes, primers and probes were previously published in the Electronic supplementary material of Monecke *et al.* [23].

Results

Among 164 Tet-R MRSA isolated between 2000 and 2011 from single patients, 33 (20%) NT_{SmaI}-MRSA strains were found. All 33 isolates belonged to ST398 and showed an *agr* type I. The first MRSA-ST398 isolate was identified in 2003. Yearly distribution of MRSA-ST398 isolates is shown in Figure 1. It is noted that out of 33 MRSA-ST398, 22 (67%) were isolated during the 2010–2011 period.

Digestions with *ApaI* restriction of MRSA-ST398 isolates provided five unrelated pulsotypes (A–E) using a cut-off at 80% similarity (Figure 2). Among these five major clusters, type A accounted for 76% (25/33) of the studied strains (Figure S1). SCC*mec* type V was carried by 29 (88%) isolates and SCC*mec* type IV was only found in four isolates. A total of six different *spa* types were detected, with t011 as the dominant type present in 23 (70%) isolates. The other *spa* types identified were: t1255 (n = 2), t1197 (n = 2), t108 (n = 2), t1451 (n = 2) and t899 (n = 1). One strain was *spa* negative both by PCR and microarray analysis (Figure 2). The BURP algorithm assigned all spa types, except singleton t899, to *Spa*-CC011 (n = 31; 94%).

The percentage of antibiotic resistance among the 33 isolates of ST398 was as follows: resistance to erythromycin was found in 11 isolates (33%), to clindamycin in 16 (48%), to tobramycin in 8 (24%) and to ciprofloxacin in 22 (67%). No resistance was found to vancomycin, daptomycin, rifampicin or mupirocin. Fourteen antibiotic resistance patterns were found among the MRSA-ST398 isolates (Table 1). The most frequent combination of resistances were: tetracycline plus ciprofloxacin (8/33; 24%) and tetracycline, ciprofloxacin, erythromycin plus clindamycin (6/33; 18%), with 49% (16/33) of the isolates being resistant to three or more antibiotic groups, in addition to beta-lactams.

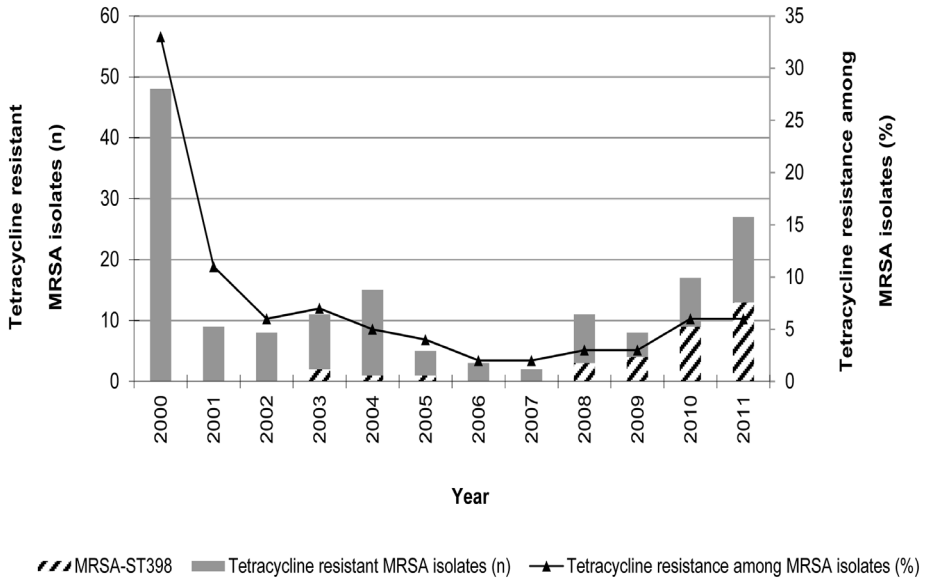
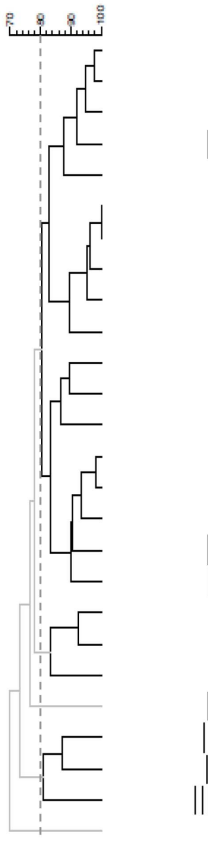


Figure 1. Distribution of 164 tetracycline resistant MRSA isolates studied from 2000 to 2011 in Hospital Universitari de Bellvitge, Barcelona, Spain.
doi:10.1371/journal.pone.0072828.g001



PFGE type (n)	MLST type	*spa* type (n)	SCC*mec* type (n)	*agr* type	Year of Isolation (n)
A 5 (5)	398	t011 (3); t108 (1); t1451 (1)	V	I	2003 (1);2004 (1); 2008 (2); 2009 (1)
A 6 (1)	398	t011 (1)	V	I	2010 (1)
A 7 (1)	398	t011 (1)	V	I	2010 (1)
A 9 (1)	398	t1255 (1)	V	I	2010 (1)
A 17 (1)	398	t011 (1)	V	I	2010 (1)
A 13 (1)	398	t011 (1)	V	I	2009 (1)
A 12 (2)	398	t011 (2)	V	I	2009 (1); 2010 (1)
A 15 (1)	398	t011 (1)	V	I	2010 (1)
A 14 (1)	398	t011 (1)	V	I	2010 (1)
A 16 (1)	398	t011 (1)	V	I	2010 (1)
A 11 (1)	398	t1255 (1)	V	I	2011 (1)
A 10 (1)	398	t011 (1)	V	I	2011 (1)
A 4 (1)	398	t011 (1)	V	I	2011 (1)
A (3)	398	t011 (2); t1451 (1)	V	I	2011 (3)
A 1 (1)	398	t011 (1)	V	I	2011 (1)
A 8 (1)	398	t011 (1)	V	I	2011 (1)
A 3 (1)	398	t011 (1)	V	I	2011 (1)
A 2 (1)	398	t108 (1)	IV	I	2011 (1)
C 2 (1)	398	t011 (1)	V	I	2011 (1)
C (1)	398	t011 (1)	V	I	2010 (1)
C 1 (1)	398	NT (1)	V	I	2010 (1)
E (1)	398	t011(1)	V	I	2003 (1)
B 2 (1)	398	t899 (1)	IV	I	2009 (1)
B (1)	398	t1197 (1)	IV	I	2005 (1)
B 1 (1)	398	t1197 (1)	IV	I	2008 (1)
D (1)	398	t011 (1)	V	I	2011 (1)

Figure 2. Cluster analysis of Pulsed-field Gel Electrophoresis (PFGE) *ApaI* macrorestriction fragments of methicillin-resistant *Staphylococcus aureus* ST398 isolates followed by multilocus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome (SCC*mec*), accessory gene regulator (*agr*) typing and year of isolation. For dendogram construction, optimization and band position tolerance were both set at 0.7%. The cut-off value to define the PFGE patterns was set at 80% similarity.
doi:10.1371/journal.pone.0072828.g002

Table 1. Antibiotic resistance patterns and resistance genes of the 33 ST398-MRSA isolates recovered in Hospital Universitari de Bellvitge, 2000–2011.

Resistance pattern[1] (n)	Tetracycline resistance genes		Other resistance genes					No. Isolates
	tet(K)	*tet*(M)	*erm*(A)	*erm*(C)	*aacA-aphD*	*aadD*	*cat*	
TET, CIP (8)	+	+	−	−	−	−	−	6
	−	+	−	−	−	−	−	2
TET (7)	+	+	−	−	−	−	−	3
	−	+	−	−	−	−	−	3
	+	−	−	−	−	−	−	1
ERY, CLI, TET, CIP (6)	+	+	−	+	−	−	−	6
CLI, TET, CIP (2)	+	+	−	−	−	−	−	2
CLI, TOB, TET, CIP (1)	+	+	−	−	−	+	−	1
ERY, CLI, GEN, TOB, TET, CIP (1)	+	+	+	+	+	−	−	1
ERY, CLI, TOB, TET, CIP(1)	+	+	−	−	−	+	−	1
CLI, GEN, TOB, TET, CIP, SYN (1)	+	+	−	−	+	+	−	1
ERY, CLI, CLO, TOB, TET (1)	+	+	−	+	−	+	+	1
ERY, CLI, TOB, TET (1)	+	+	−	+	−	+	−	1
ERY, CLI, TOB, TET, SXT, CIP (1)	+	+	−	+	−	+	−	1
TOB, TET, CIP (1)	−	+	−	−	−	+	−	1
CLI, TET (1)	+	+	−	−	−	−	−	1
CLO, TET (1)	+	+	−	−	−	−	+	1
Total No of Isolates								**33**

[1]TET, tetracycline; CIP, ciprofloxacin; CLI, clindamycin; TOB, tobramycin; GEN, gentamicin; SYN, synercid; ERY, erythromycin; CLO, chloramphenicol; SXT, sulfamethoxazole-trimethoprim.

doi:10.1371/journal.pone.0072828.t001

Antibiotic resistance genes, as determined by DNA hybridization, are shown in Table 1. All 33 strains harboured *mec*A and the beta-lactamase gene *bla*Z. Tetracycline resistance in most of the cases (26/33) was mediated by both *tet(*K) and *tet(*M) genes. In addition to tetracycline resistance, erythromycin and clindamycin combined resistance was seen in 10 (30%) isolates carrying the gene *erm*(C)– one of these isolates harbored *erm*(A) in addition to *erm*(C)-, and one isolate was negative for these genes. Other macrolide and lincosamide resistance genes reported to occur in staphylococci such as *erm*(B), *msr*(A), *mph*(C), *vga*(A) and *lnu*(A) were negative in this subgroup of strains. Five isolates (15%) showed an unusual erythromycin-susceptibility/clindamycin-resistance pattern. No genes associated with this resistance phenotype such as *vga*(A) or *lnu*(A) were identified by microarray in the MRSA-ST398 strains studied. Two isolates showing resistance to gentamicin harbored the gene *aacA/aphD*. Tobramycin resistance was mediated by *aacA/aphD* (1/8) and by *aadD* (7/8) genes. Chloramphenicol resistance was detected in 2 strains carrying the *cat* gene.

Regarding the presence of genes coding for virulence factors, none of the MRSA-ST398 isolates harbored the genes encoding PVL (*luKS-PV/ lukF-PV*), enterotoxins (*sea* to *ser*), exfoliative toxins (*etA/etB/etD*), *egc* cluster (*seg/sei/sem/sen/seo/seu*) or TSST (*tst*). A single isolate carried the *seb* gene encoding enterotoxin B. The vast majority of the strains were positive for haemolysins genes *hla*, *hlb*, *hld*, *hlgA*, *hlgB* and *hlgC*. All but one, were positive for the *cna* gene responsible by the collagen-binding adhesion. Genes carried on mobile genetic elements and involved in immune evasion such as *scn* (staphylococcal complement inhibitor), *sak* (staphylokinase) and *chp* (chemotaxis inhibitory protein) were identified in a single isolate of *spa* type t899. Fibronectin-binding protein A gene (*fbnA*) was detected in 9/33 (27%) of the isolates. All isolates carried genes codifying capsule type 5.

The clinical characteristics of patients infected or colonized by MRSA-ST398 are shown in Table 2. In total, there were 27 men (82%) and 6 women (18%) with a mean age of 65 years (range 41 to 92). In 23 patients (70%), the isolation of MRSA-ST398 was considered colonization, more frequently as result of active surveillance for nasal MRSA colonization (17/23; 78%). MRSA-ST398 was causing infection in 10 patients. Eight patients experienced non-invasive infections: in four cases the source of the infection was the respiratory tract and in the other four cases the patients experienced non-invasive skin and soft tissue (SST) infections. Two patients, with prior community-acquired MRSA-ST398 nasal colonization, went through invasive infections: one of them suffered a catheter-related bacteremia and the other a subdural empyema following a surgical drainage of a subdural haemorrhage. The patient with the catheter-related bacteremia was the sole case where death could be related to the MRSA-ST398 infection. The most frequent underlying disease was cancer, with this condition found in 11 patients, of whom 6 were colonized and 5 were infected by MRSA-ST398.

Discussion

Since 2005, several studies have been carried out focusing on livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) isolates, mainly on isolates of CC398 [1,3–5,10,13,24,25]. Human colonization with LA-MRSA was first described among swine farmers in France and The Netherlands in 2003 [4,9]. This is the first retrospective study of MRSA-ST398 in Spain covering a period from 2000 to 2011. In order to evaluate the prevalence and

Table 2. Clinical features of 33 patients colonized or infected by ST398-MRSA.

	No. (%) or mean ± SD with variable	
Men	27 (81)	
Age, years (range)	65 (41–92)±12.9	
Rural area	24 (77)	
Underlying conditions		
Cancer	11 (33)	
Sample Source	**Colonization**	**Infection**
Blood	0 (0)	1 (3)
Respiratory tract	1 (3)	4 (12)
Skin and Wound	4 (12)	4 (12)
Nares	18 (55)	0 (0)
Central Nervous System	0 (0)	1 (3)
Total number (%)	23 (70)	10 (30)

doi:10.1371/journal.pone.0072828.t002

molecular characteristics of this clone in our hospital, one hundred and sixty-four isolates obtained between 2000 and 2011 from different sources were investigated. Prior to year 2000 Tet-R was very high among the MRSA isolated from the patients admitted to the HUB. This was due to the presence of an endemic clone – the Iberian clone (ST247) that was resistant to several antibiotics, including tetracycline. In the following years, tetracycline resistance dropped, often below 5%. Antibiotic susceptibility analysis revealed an unexpected diversity of resistance profiles among MRSA-ST398 isolates. In total, 14 different resistance patterns were seen with 10 of them represented by only one or two isolates. Of note, 49% of the isolates exhibited resistance to three or more antimicrobial agents, in addition to methicillin-resistance. Ciprofloxacin resistance was high (67%) considering the low percentage of resistance to this antibiotic exhibited by other MRSA community clones. Spanish [7] and Belgian [5] studies described resistance to fluoroquinolones of 58% and 83% respectively. However, in other European countries [14], USA [10] or Canadian studies [26], the resistance percentage to fluoroquinolones is very low or even inexistent. The veterinary use of quinolones in Spain is difficult to assess. The public data available on the website of the Spanish Ministry of Health (*Ministerio de Sanidad, Politica Social y Igualdad*) (www.aemps.gob.es) only describes the total amount of antimicrobials sold for veterinary purposes, and does not distinguish between the therapeutic use of antibiotics and the use of antibiotics to enhance growth. In any case, total sales of quinolones was about 50 tons in 2009, much lower than the sales of other antibiotics such as tetracyclines (350 tons), sulfonamides (250 tons) or beta-lactams (180 tons) during the same year.

The combination of *tet*(K) and *tet*(M) resistance genes was commonly seen among the 33 MRSA-ST398 isolates. The methylase *erm*(C) gene was present in 10 out of 11 erythromycin resistant strains. Resistance to cotrimoxazole was found in a single isolate, whereas in other MRSA-ST398 studies cotrimoxazole resistance was more prevalent and in some cases exceeded 80% [5,27].

Some strains showed the unusual erythromycin-susceptibility/clindamycin-resistance pattern. This phenotype seems to be related to animal clonal lineages of *S. aureus* animals and has been associated with the presence of plasmid-borne resistance genes *vga*(A), *vga*(C), *lnu*(A) or *lnu*(B) [28,29]. Some resistance genes described in MRSA CC398 strains such as *erm*(T), *vga*(B), *vga*(C), *lnu*(B), *dfrSI, dfrK, dfrG* and *tet*(L) [27] were not included in the DNA microarray applied in this study.

The dominant *spa* type t011 in our collection has been commonly found in other studies, both in Spain [7], and in other countries [8,25,30,31]. There was a single isolate of *spa* type t899, with only two repeats in common with t011. Therefore, this t899 isolate did not cluster in *Spa*-CC011 after BURP analysis. As described previously [30], this could be explained by the acquisition of a large DNA region which includes the *spa* gene, from a remote *S. aureus* clone. *spa* type t899 has been described in association to ST9, a clone also described in samples of animal origin [32]. The linkage of the same *spa* type in two different ST types would suggest exchange of genetic material between two clones of animal origin.

The presence of genes coding for virulence factors was very poor in our collection. None of the studied isolates carried the PVL – encoding genes *lukF-PV* and *lukS-PV*, contrasting to studies from Sweden and China that report PVL-positive isolates in patients who had no previous contact with animals [6,11]. Generally, MRSA-ST398 lacks certain important virulence factors for humans [13]. In our study genes involved in immune evasion (*scn*, *sak* and *chp*) were only detected in the single t899 isolate. These virulence factors are active only against the innate immune system in humans [33]. The lack of these virulence factors may partially explain why these strains do not appear to be highly infectious for humans, and usually are associated with SST infections [25]. However, a few severe infections by ST398 have been sporadically published in several countries, such as pneumonia or bacteraemia [24,31]. Even though the majority of the patients in our study were colonized by MRSA-ST398 (70%), four SST infections and four respiratory tract infections were detected in our series. In addition, two invasive infections, bacteraemia and subdural empyema, were also detected, in patients showing previous nasal colonization. Death was related to the MRSA-ST398 infection in the bacteremic patient: an 84-year-old woman who was hospitalized in the HUB because of a thoracic aortic aneurysm. One month after the surgery, the patient experienced a febrile episode and MRSA-ST398 was recovered from blood and from the central venous catheter tip. The isolate was resistant to tetracycline and beta-lactams. One week after treatment with vancomycin, the patient died. The isolates causing infection did not differ from the colonizing isolates, regarding genotype, virulence or antibiotic resistance profile.

In our study, some data could not be collected properly such as the contact with animals, a risk factor for infection by LA-MRSA. However, a high percentage (73%) of the patients lived in or near to a rural environment. Another limitation was the selection of the isolates to be studied by the presence of tetracycline resistance. Although this is a common feature among MRSA-ST398 [7], this approach could underestimate the number of isolates belonging to CC-398 in HUB.

In conclusion, in the last two years (2010–11) the number of MRSA-ST398 isolates infecting or colonizing patients increased significantly in our setting, as well as the increase in tetracycline resistance. The emergence of this clonal lineage has also been reported in other countries in Europe [8,24,25,34]. These studies showed a remarkable increase in the proportion of LA-MRSA isolates, including outpatients and primary health care patients, which were not covered in our hospital-based study. According to our results, the majority of studied isolates carried the genes encoding haemolysins and adhesion cellular factors, but other virulence factors usually found among *S. aureus* were not detected.

Phenotypic expression of antibiotic resistance was variable among the MRSA-ST398 isolates and nearly half of the isolates were resistant to multiple antibiotics. In addition, patients harbouring this clone were often debilitated by underlying diseases, such as cancer. Due to the increased public health interest about MRSA-ST398, further studies should be conducted to record risk factors from infected or colonized patients by this lineage such as routes of transmission and association with animals.

Supporting Information

Figure S1 Pulsed-field Gel Electrophoresis (PFGE) of *ApaI* macrorestriction fragments of methicillin-resistant *Staphylococcus aureus* ST398 isolates showing a PFGE pattern A5 followed by multilocus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome (SCC*mec*), accessory gene regulator (*agr*) typing and year of isolation data. For dendogram construction, optimization and band position tolerance were both set at 0.7%. The cut-off value for designing genotypes was set at 80%.
(TIF)

Acknowledgments

These data were presented at the 22nd European Congress of Clinical Microbiology and Infectious Diseases, London, UK, 2012 (communication no.O270).

Author Contributions

Conceived and designed the experiments: MC MAD. Performed the experiments: MC. Analyzed the data: MC JMS MP AH RM MAD. Contributed reagents/materials/analysis tools: MC JMS MP AH RM MAD. Wrote the paper: MC MAD.

References

1. Stegger M, Lindsay JA, Sorum M, Gould KA, Skov R (2010) Genetic diversity in CC398 methicillin-resistant *Staphylococcus aureus* isolates of different geographical origin. Clin Microbiol Infect 16: 1017–1019.
2. Argudin MA, Fetsch A, Tenhagen BA, Hammerl JA, Hertwig S, et al. (2010) High heterogeneity within methicillin-resistant *Staphylococcus aureus* ST398 isolates, defined by *Cfr9*I macrorestriction-pulsed-field gel electrophoresis profiles and spa and SCC*mec* types. Appl Environ Microbiol 76: 652–658.
3. Graveland H, Wagenaar JA, Bergs K, Heesterbeek H, Heederik D (2011) Persistence of livestock associated MRSA CC398 in humans is dependent on intensity of animal contact. PLoS One 6: e16830.
4. Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M (2005) Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis 11: 1965–1966.
5. Denis O, Suetens C, Hallin M, Catry B, Ramboer I, et al. (2009) Methicillin-resistant *Staphylococcus aureus* ST398 in swine farm personnel, Belgium. Emerg Infect Dis 15: 1098–1101.
6. Welinder-Olsson C, Floren-Johansson K, Larsson L, Oberg S, Karlsson L, et al. (2008) Infection with Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* t034. Emerg Infect Dis 14: 1271–1272.
7. Lozano C, Rezusta A, Gomez P, Gomez-Sanz E, Baez N, et al. (2012) 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 67: 330–334.
8. van Cleef BA, Monnet DL, Voss A, Krziwanek K, Allerberger F, et al. (2011) Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis 17: 502–505.
9. Armand-Lefevre L, Ruimy R, Andremont A (2005) Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg Infect Dis 11: 711–714.
10. Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, et al. (2009) Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One 4: e4258.
11. Yu F, Chen Z, Liu C, Zhang X, Lin X, et al. (2008) Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin Microbiol Infect 14: 381–384.
12. Aspiroz C, Lozano C, Vindel A, Lasarte JJ, Zarazaga M, et al. (2010) Skin lesion caused by ST398 and ST1 MRSA, Spain. Emerg Infect Dis 16: 157–159.
13. Kadlec K, Ehricht R, Monecke S, Steinacker U, Kaspar H, et al. (2009) Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. J Antimicrob Chemother 64: 1156–1164.
14. Pan A, Battisti A, Zoncada A, Bernieri F, Boldini M, et al. (2009) Community-acquired methicillin-resistant *Staphylococcus aureus* ST398 infection, Italy. Emerg Infect Dis 15: 845–847.
15. CLSI (2010) Performance standards for antimicrobial susceptibility testing; 20th Informational Supplement. CLSI document M100-S20. Wayne, pa: Clinical and laboratory standards institute.
16. Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, et al. (2000) Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb Drug Resist 6: 189–198.
17. van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, et al. (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13 Suppl 3: 1–46.
18. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol 38: 1008–1015.
19. Lina G, Boutite F, Tristan A, Bes M, Etienne J, et al. (2003) Bacterial competition for human nasal cavity colonization: role of Staphylococcal *agr* alleles. Appl Environ Microbiol 69: 18–23.
20. Milheirico C, Oliveira DC, de Lencastre H (2007) Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob Agents Chemother 51: 3374–3377.
21. Strommenger B, Braulke C, Heuck D, Schmidt C, Pasemann B, et al. (2008) *spa* Typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J Clin Microbiol 46: 574–581.
22. Monecke S, Slickers P, Ehricht R (2008) Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. FEMS Immunol Med Microbiol 53: 237–251.
23. Monecke S, Berger-Bachi B, Coombs G, Holmes A, Kay I, et al. (2007) Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. Clin Microbiol Infect 13: 236–249.
24. van Belkum A, Melles DC, Peeters JK, van Leeuwen WB, van Duijkeren, et al. (2008) Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg Infect Dis 14: 479–483.
25. van Loo I, Huijsdens X, Tiemersma E, de Neeling A, van de Sande-Bruinsma N, et al. (2007) Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg Infect Dis 13: 1834–1839.
26. Golding GR, Bryden L, Levett PN, McDonald RR, Wong A, et al. (2010) Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 in humans, Canada. Emerg Infect Dis 16: 587–594.
27. Cuny C, Nathaus R, Layer F, Strommenger B, Altmann D., et al. (2009) Nasal colonization of humans with methicillin resistant *Staphylococcus aureus* (MRSA) CC398 with and without exposure to pigs. PLoS One 4: e6800.
28. Kadlec K, Fessler AT, Hauschild T, Schwarz S (2012) Novel and uncommon antimicrobial resistance genes in livestock-associated methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect 18: 745–755.
29. Lozano C, Aspiroz C, Saenz Y, Ruiz-Garcia M, Royo-Garcia G, et al. (2012) Genetic environment and location of the *lnu*(A) and *lnu*(B) genes in methicillin-resistant *Staphylococcus aureus* and other staphylococci of animal and human origin. J Antimicrob Chemother 67: 2804–2808.
30. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, et al. (2012) *Staphylococcus aureus* CC398: host adaptation and emergence of methicillin resistance in livestock. MBio 4(1): e00520–12.
31. Witte W, Strommenger B, Stanek C, Cuny C (2007) Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg Infect Dis 13: 255–258.
32. Lo YP, Wan MT, Chen MM, Su HY, Lauderdale TL, et al. (2012) Molecular characterization and clonal genetic diversity of methicillin-resistant *Staphylococcus aureus* of pig origin in Taiwan. Comp Immunol Microbiol Infect Dis 35(6): 513–521.
33. Rooijakkers S, van Kessel KPM, van Strijp JAG (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596–601.
34. Schaumburg F, Köck R, Mellmann A, Richter L, Hasenberg F, et al. (2012) Population dynamics among methicillin-resistant *Staphylococcus aureus* isolates in Germany during a 6-year period. J Clin Microbiol 50(10): 3186–3192.

Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Barcelona, Spain, 2012-2013.

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Abstract

Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 (MRSA-ST398) has been described as a new clonal lineage able to colonize and infect humans in several countries around the world. The increased rate of this clone from 2010 to 2011 in our setting, lead us to investigate the prevalence and the molecular features of MRSA-ST398 isolates, identified from 2012 to 2013. All MRSA strains were screened by *Sma*I-PFGE. Nontypable MRSA strains by *Sma*I (NT-*Sma*I) were re-analyzed by *Apa*I-PFGE. Molecular typing including SCC*mec, agr,* MLST and DNA microarray hybridization were performed for all NT-*Sma*I isolates. Molecular characterization revealed that 16 of 1,127 (1.4%) MRSA strains isolated during this 2-year period belonged to the MRSA-ST398 clone. The majority of the MRSA-ST398 isolates belonged to PFGE type A (56%), carried SCC*mec* V (94%) and was *spa* type t011 (75%). Most of the strains revealed resistance to ciprofloxacin (81%) and low virulence genes content was observed. All MRSA-ST398 isolates in this study had a variety of genes encoding proteins associated with biofilm formation. Colonization or infection by this clone occurred most often in debilitated patients. Three cases were hospital-acquired. Our study shows that the MRSA-ST398 clone was present in low numbers in the Southern Barcelona metropolitan area. Colonization was mainly of community origin and in some patients, persisted for a long period. Of note, hospital transmission was hypothesized in a few cases. We report two MRSA-ST398 isolates showing the genetic markers suggesting being associated with a human adapted ST398 phenotype.

Keywords: Methicillin-resistant *Staphylococcus aureus*; Livestock-associated-MRSA; Tetracycline resistance; ST398; Spain.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) of clonal complex (CC) 398 so-called livestock-associated MRSA (LA-MRSA), emerged as a new clonal lineage infecting or colonizing humans worldwide [1–4]. It has been suggested that a human MSSA CC398 lineage jump and spread to livestock host, where it acquired the SCC*mec* cassette and *tet*(M) gene encoding methicillin and tetracycline resistance (Tet-R) respectively [5]. On the other hand, the LA-CC398 clone tipically lacks proteins that are involved in human-specific innate immune system such as the staphylococcal complement inhibitor, encoded by the *scn* gene, harboured in the immune evasion cluster (IEC) region. LA-CC398 clustered into two major distinct phylogenetic clades: a basal human clade and a more derived livestock clade [5, 6]. The absence of *tet*(M) gene is associated with the human-adapted clade and the loss of the immune evasion cluster (IEC) genes harbouring *scn* gene is associated with the livestock-adapted clade [5].

Two main microbiological features have been described in MRSA-ST398 isolates: resistance to tetracycline [7,8], and inability to be restricted by *Sma*I enzyme (NT_{SmaI}-MRSA) [9].

Several studies reported that close animal contact increased the risk of being colonized or infected with MRSA-CC398 [10–12]. However, discordant findings were revealed about human persistent colonization and transmission routes between human and animals [13,14].

MRSA strains with sequence type (ST) 398 are mostly associated to skin and soft tissue infections (SSTIs) [15]. Although it has been suggested that MRSA-ST398 is less virulent than other human MRSA clones, due to the lack of important toxins such as Panton-Valentine leukocidin (PVL) and other enterotoxins [16], severe invasive infections such as pneumonia, bacteraemia and endocarditis [17–19] have been reported. Transmission of MRSA-ST398 has also been reported in a nosocomial outbreak in the Netherlands [7].

First descriptions of human colonization by MRSA-ST398 were reported in the Netherlands and France in mid 2000s [11,20]. Since then, this clone has been reported in several European countries, but most cases were located in Belgium, Denmark, Austria and the Netherlands [1]. In Spain, previous studies have shown the presence of MRSA-ST398. However, high rates of this clonal type have only been detected in certain areas with higher density of pig farming [5,15,21].

A retrospective study carried out in our setting from 2000 to 2011 identified the first MRSA-ST398 isolate in 2003 [22]. The number of cases was scarce until the 2010-2011 period when half of the tetracycline resistant MRSA isolates belonged to the ST398 clone [22]. This fact and the identification of two MRSA-ST398 led us to monitor the MRSA-ST398 clone in the southern metropolitan area in Barcelona and to update the epidemiology and microbiology features of this clone among the most recent isolates from the 2012-2013 period.

Material and methods

Source of bacterial isolates and setting

The present study has been approved by the Clinical Research Ethics Committee of the Hospital Universitari de Bellvitge. The Ethics Committee granted an exemption on obtaining the patients' informed consent as this retrospective study focused on bacteria characterization and did not require any specific patient involvement. All patient records/information was anonymized and de-identified prior to analysis.

This study has been performed at Hospital Universitari de Bellvitge (HUB), a university hospital providing tertiary health care with approximately 700 beds in the Barcelona southern metropolitan area. The hospital belongs to the Institut Català de la Salut, the main public health supplier in Catalonia, Spain. The HUB is a referral centre for adult patients with approximately 30,316 admissions per year, serving a population of 1,000,000 inhabitants in both urban and rural areas.

From January, 2012 to December, 2013 the overall rate of MRSA among clinical *S. aureus* strains isolated in our setting was 25%. The Microbiology Department of the HUB identified 1,127 MRSA isolates from individual patients during the study period. This number included isolates from clinical samples (n= 472), and isolates from active surveillance culture samples (n= 655). Clinical data of patients infected or colonized by MRSA of ST398 were reviewed.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by automatized microdilution in invasive and clinical MRSA isolates using the MicroScan system (MicroScan® - Pos Combo Panel Type 31, Siemens AG, Germany). The disk diffusion method was applied, in accordance to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [23], to the nasal MRSA isolates obtained from active surveillance cultures. The following antibiotics were tested: penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, gentamicin, tobramycin, rifampicin, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, ciprofloxacin, vancomycin, teicoplanin, mupirocin quinupristin/dalfopristin, and linezolid.

Molecular characterization

In order to monitor the presence of MRSA-ST398 in our setting, all 1,127 MRSA isolates were screened by *Sma*I-pulsed field gel electrophoresis (PFGE) according to Chung et al. [24]. The MRSA isolates that could not be typed by *Sma*I (NT_{SmaI}-MRSA) were re-analysed by *Apa*I-PFGE as described previously [22]. The resulting restriction patterns by *Apa*I enzyme, were interpreted both by visual inspection, using the criteria of van Belkum et al. [25], and by analysis with the FINGERPRINTING TM II software, version 3.0 (BioRad Laboratories, Inc., Madrid, Spain). PFGE patterns were normalized using the lambda ladder marker (New England Biolabs Inc., Beverly, MA, USA) and was included in two

lanes of the 18-lane gel. The dendogram was generated using the unweighted-pair group with arithmetic averages method based on Jaccard coefficients, where optimization and band position tolerance were set at 0% and 0.7% respectively. PFGE type clusters were defined by a similarity coefficient of 80%. Multilocus sequence typing (MLST) was performed according to Enright et al. [26] on representative isolates of each *Apa*I-PFGE type and sequence types (ST) were assigned using *S. aureus* MLST database (http://www.mlst.net). Further molecular analysis was performed for all NT_{SmaI}-MRSA isolates. The detection of *agr* polymorphism subtypes (*agr*I to IV) [27] and the Staphylococcal cassette chromosome *mec* (*SCCmec*) typing [28] were carried out by multiplex PCR strategy. Strains were characterized by *spa* typing [29] using the Ridom StaphType software, version 1.4 (Ridom GmbH Münster, Germany) and according to the nomenclature as described on the Ridom website (http://www.spaserver.ridom.de). Clonal relatedness between *spa* types (*spa*-CC) was calculated by the BURP algorithm applying the default parameters set by the software. Virulence genes and resistance encoding genes were characterized using the DNA microarray Identibac MRSA Array-Strip (Staphy-Type Kit, Alere Technologies Ltd, Jena, Germany; stripe version), according to the manufacturer's instructions extensively described elsewhere [30].

Results

Of the 1,127 MRSA strains isolated during the 2012-2013 period from single patients, 16 (1.4%) NT_{SmaI}-MRSA isolates were found. All 16 isolates share MLST ST398 and *agr* type I.

Six unrelated pulsotypes (A-F) were provided by *Apa*I-PFGE restriction, with type A accounting 56% (9/16) of the strains (Fig. S1). All isolates carried the SCC*mec* type V with the exception of one isolate that carried the SCC*mec* type IV. Further analysis by *spa* typing revealed 4 different types, with t011 as the most frequent type (n=12; 75%), followed by t1451 (n=2; 13%), t5524 (n=1; 6%) and t899 (n=1; 6%). The BURP cluster analysis assigned all *spa* types, except singleton t899, to *spa*-CC011 (n=15; 94%).

Susceptibility testing identified that all MRSA-ST398 isolates were resistant to tetracycline, 81% (13/16) to ciprofloxacin, 38% (6/16) to clindamycin, 31% (5/16) to erythromycin and 25% (4/16) to tobramycin. All isolates were susceptible to glycopeptides, daptomycin, rifampin and mupirocin. Six different antibiotic resistance patterns were found among the MRSA-ST398 isolates (Table 1), the most common one being the combination of resistance to tetracycline plus ciprofloxacin (8/16; 50%). By DNA microarray Tet-R was (12/16) mediated by both *tet*(M) and *tet*(K) genes in 12 isolates (12/16; 75%) (Table 1). All the strains carried *mec*A and harbored the beta-lactamase gene *bla*Z. Combined resistance to macrolides-lincosamides was detected in 5 isolates carrying the *erm*(C) (4/5) and *erm*(C) plus *erm*(B) (1/5) genes. A single isolate showed an uncommon erythromycin-susceptible/clindamycin resistance profile with the absence of typical genes associated to this phenotype such as *vga*(A) or *lnu*(A). Gentamicin resistance was detected in two isolates: one carried the *aacA-aphD* gene and the other one carried both *aacA-aphD* and *aadD* genes.

In four isolates tobramycin resistance was mediated by *aacA-aphD* (1/4), *aacA-aphD* plus *aadD* (1/4), and *aadD* (2/4) genes. The *czr*C, cadmium and zinc

185 resistance genes, were identified in all ST398 isolates carrying the SCC*mec*
186 type V.

187 Concerning the virulence content of MRSA-ST398 isolates, DNA
188 hybridization was negative for genes encoding PVL (*lukS-PV*/*lukF-PV*),
189 enterotoxins (*sea* to *ser*), exfoliative toxins (*etA*/*etB*/*etD*), *egc* cluster
190 (*seg*/*sei*/*sem*/*seo*/*seu*) or TSST (*tst*). All the strains were positive for the
191 haemolysins genes *hla*, *hlb*, *hld*, *hlgA*, *hlgB* and *hlgC* and carried genes
192 codifying capsule 5 *(capH5, capJ5, capK5).*

193 The biofilm formation and MSCRAMM (microbial surface components
194 recognizing adhesive matrix molecules) carriage genes was similar among all
195 isolates including *icaACD* (intercellular adhesion biofilm genes A, C, and D),
196 *cflA* and *cflB* (clumping factors A and B), *cna* (collagen-binding protein), *fn*bA
197 and f*nbB* (fibronectin-binding proteins A and B). Additionally, our study revealed
198 that all isolates lacked *sasG* (*S. aureus* surface protein G) gene and 13 isolates
199 (13/16; 81%) were positive for the *bbp* (bone sialoprotein-binding protein) gene.
200 Two isolates carried genes encoding proteins that were involved in human-
201 specific innate immune system such as staphylococcal complement inhibitor
202 (*scn*), chemotaxis inhibitory protein (*chp*) and staphylokinase (*sak*) (Table 1).

203 Clinical features of the 16 patients colonized or infected by MRSA-
204 ST398 were reviewed. No significant difference between gender was observed
205 (man, n=9; women, n=7) and the mean age was 58 years (range 33 to 81).
206 Fourteen patients underwent surgical procedures during admission. The most
207 common underlying disease was cancer (7/16; 44%). In 10 patients (10/16;
208 63%), MRSA-ST398 was associated with colonization. Six patients (6/16; 38%)
209 were infected: four experienced non-invasive SSTIs, one a bacteremia and the

other one a surgical bone infection (Table 2). Prior nasal colonization by MRSA-ST398 was detected in four infected patients (two SSTIs, one bacteremia and one bone infection). Three cases (two SSTIs and one colonization detected by tracheostomy swab) were hospital acquired, since all three had the nasal swab taken at admission negative for MRSA. No epidemiological links could be identified among the three patients with hospital acquired strains. In the remaining 13 cases, acquisition of colonization was ascribed to the community.

In five patients (two with SSTIs and three showing only nasal colonization), nasal swabs were repeatedly positive for MRSA-ST398 for a mean period of 25 months (range 5-66 months) (Table 2).Compared to the 2010-2011 period when 48% (22/46) of the Tet-R MRSA belonged to clone MRSA-ST398, the current period of study 2012-2013 showed a similar proportion of Tet-R MRSA belonging to ST398: 37% (16/43) . Referred to the total number of MRSA isolates, in the 2010-2011 period MRSA-ST398 represented 2.0 % (22/1119) of MRSA isolates. This proportion in the 2012-2013 period was 1.4% (16/1127).

Discussion

In the last decade, several studies have been carried out warning of the international emergence of MRSA-ST398 clone among humans [1,2,10,11]. These studies revealed a significant increase of the livestock-associated MRSA prevalence among outpatients and primary health care patients. Recently, a multicentre study carried out in Spain, described the increased rate of MRSA-

ST398 among CA-MRSA during 2012 [21]. We described a similar trend in our clinical setting, where MRSA-ST398, in spite of low numbers, represented 48% of tetracycline resistant MRSA strains isolated between 2010 and 2011 [22]. However, we find a similar proportion of MRSA-ST398, 37%, during the 2012-2013 period, representing 1.4% of all MRSA. Higher proportions of MRSA-ST398 among MRSA clinical isolates (5%) were described in a different Spanish region [15], as well as in other European countries that also carried out their studies in areas with high levels of livestock production, i.e. Netherlands 11.9% or Belgium 4.7% [1].

Most of the MRSA-ST398 isolates were *spa* type t011 which is the predominant *spa* type among pig-associated isolates [10] often reported in Spain [15,22] and other countries [1, 5,10,17]. A single isolate was ascribed to *spa* type t899 and did not cluster in *spa*-CC011 by BURP analysis. Price *et al*. reported that the strains carrying *spa* type t899 did not follow the whole genome sequencing phylogeny, and this fact could be explained by the acquisition of a large DNA region including SCC*mec* and the *spa* gene from a ST9 donor [5]. The linkage of the same *spa* type in two different ST types would suggest exchange of genetic material between two clones of animal origin. All isolates but one carried the SCC*mec* type V. It has been suggested that the use of zinc and others metals in animal feed formulations, can co-select SCC*mec* type V, carrying the *czrC* zinc resistance gene [31].

Antibiotic susceptibility analysis provided evidence of a variety of resistance profiles among MRSA-ST398 isolates. The diversity of antimicrobial resistance phenotypes and genotypes among MRSA-CC398 strains has been reported worldwide and results from the capability of this lineage to acquire

external DNA combined with the antimicrobial selection pressure applied to the microorganisms in diverse environments [32,33]. Of note, ciprofloxacin resistance was detected in 13 out of 16 isolates (81%), in contrast to other MRSA community clones which exhibit a low resistance rate to this antibiotic [21]. All MRSA-CC398 were Tet-R, this fact do reflect the antimicrobial veterinary use in our country. The European Medicines Agency reported Spain to be the second country consuming veterinary antimicrobial agents in 2012, with sales of tetracyclines, penicillins, macrolides, lincosamides, and polymyxins accounting for 82.7% and quinolones accounting for 3.4% of the various veterinary antimicrobial classes sold in Spain during 2012 [34].

Resistance to macrolides-lincosamides was mainly mediated by *erm*(C) gene. An unusual erythromycin-susceptibility/clindamycin-resistance phenotype was detected in a single isolate. Our strain lacked *vga*(A) and *lnu*(A) genes. However, other genes such as *vga*(C) and *lnu*(B) that could be associated to this phenotype [32,35] were not tested in our study.

In the context of the CC398, the absence of *tet*(M) has been considered a marker of human origin [5]. In our study, the single isolate (patient number 12) carrying only *tet*(K) and lacking *tet*(M), could thus be derived from a human-associated lineage [5,36]. Interestingly, only two isolates (patient numbers 7 and 12) presented the genes encoding the IEC protein complexes (*scn*, *chp* and *sak*) located on φ3 bacteriophages that are exclusively carried in human CC398 isolates [37,38]. In contrast to a previous study, the IEC genes were not only detected in an invasive isolate but also in a human colonization isolate, suggesting that these genes they don´t play an essential role in invasive disease [38]. Molecular findings could suggest that these two isolates belonged

to two different clades: one carrying both ICE and *tet*(M) genes (patient number 7) and other (patient number 12) only carrying the ICE genes [36]. However, further in-depth analysis is needed to determine whether the two aberrant strains belong to the human- or livestock-adapted CC398 lineage. Moreover, the low frequency of these IEC genes indicates that they are not crucial for human adaptation of CC398 and could help to understand why these strains are less virulent in humans than other clone lineages and are most commonly associated with SST infections [5,10,38]. In general, this MRSA-ST398 clone typically lacks important virulence genes, including the PVL gene [7,33], which is in accordance with our results. Only a few studies reported PVL-positive strains isolated from patients with or without previous contact with livestock [3,39,40]. Moreover, the majority of the studied MRSA-ST398 isolates shared the same genes for biofilm production and genes encoding MSCRAMMs and were similar to those described from other CC398 strains [41,42].

The phenotype and genotype of isolates causing infection in our study did not significantly diverge from the colonizing isolates. The patients carrying MRSA-ST398 in HUB were admitted to the hospital to undergo surgical procedures, to be treated for an underlying disease (mainly cancer or cardiovascular dysfunction) or both. In four infected patients, colonization by the MRSA-ST398 was detected at admission prior to the development of the infection. Two more patients acquired the infection while admitted to the hospital. However, no other nosocomial transmitted cases were generated, and no carriers were found among hospital staff. Also of note is the persistent carriage found in some patients and in one case the nasal colonization was invariably detected for more than five years.

Limitation to our study was the difficulty in assessing the patients exposure to animals, a risk factor for carriage of MRSA-ST398 [13]. However, colonization or infection by MRSA-ST398 have been reported in persons lacking identified livestock-associated risk factors [4,7].

In conclusion, our study shows that the MRSA-ST398 clone was present in the Southern Barcelona Metropolitan area, although in low numbers, and patients attending the hospital showed colonization mainly of community origin. In three cases (two infections and one tracheostomy colonization), hospital transmission was hypothesized since the patients were repeatedly negative for nasal carriage, prior to the isolation of the MRSA-ST398 from a clinical sample. Interestingly, genetic analysis revealed two MRSA-ST398 isolates with molecular patterns of human re-adaptation.

Acknowledgements

These data were partially presented at the 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, ES 2014 (communication no. 1659).

This study was funded by a grant from Fondo de Investigación Sanitaria (reference PI 10/01783). Mariana Camoez was supported by Plan Nacional de I+D+i 2008-2011 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD12/0015) - co-financed by European Development Regional Fund ‘‘A way to achieve Europe’’ ERDF.

References

1. van Cleef BA, Monnet DL, Voss A, Krziwanek K, Allerberger F, Struelens M, Zemlickova H, Skov RL, Vuopio-Varkila J, Cuny C, Friedrich AW, Spiliopoulou I, Pászti J, Hardardottir H, Rossney A, Pan A, Pantosti A, Borg M, Grundmann H, Mueller-Premru M, Olsson-Liljequist B, Widmer A, Harbarth S, Schweiger A, Unal S, Kluytmans JAJW (2011) Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg Infect Dis 17:502–505.

2. Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, Moritz ED, Capuano AW, Herwaldt LA, Diekema DJ (2009) Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One 4:e4258.

3. Yu F, Chen Z, Liu C, Zhang X, Lin X, Chi S, Zhou T, Chen Z, Chen X (2008) Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin. Microbiol. Infect 14:381–384.

4. Golding GR, Bryden L, Levett PN, McDonald RR, Wong A, Wylie J, Graham MR, Tyler S, Domselar GV, Simor AE, Gravel D, Mulvey MR (2010) Livestock-associated methicillin-resistant *Staphylococcus aureus* sequence type 398 in humans, Canada. Emerg. Infect. Dis 16:587–594.

5. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen PS, Pearson T, Waters AE, Foster JT, Schupp J, Gillece J, Driebe E, Liu CM,

354 Springer B, Zdovc I, Battisti A, Franco A, Zmudzki J, Schwarz S, Butaye
355 P, Jouy E, Pomba C, Porrero MC, Ruimy R, Smith TC, Robinson DA,
356 Weese JS, Arriola CS, Yu F, Laurent F,m Keim P, Skov R, Aarestrup FM
357 (2012) *Staphylococcus aureus* CC398: host adaptation and emergence of
358 methicillin resistance in livestock. MBio 3:e00305–11.

359 6. McCarthy AJ, van Wamel W, Vandendriessche S, Larsen J, Denis O,
360 Garcia-Graells C, Uhlemann AC, Lowy FD, Skov R, Lindsay JA (2012)
361 *Staphylococcus aureus* CC398 clade associated with human-to-human
362 transmission. Appl Environ Microbiol 78:8845-8848.

363 7. Wulf MW H, Markestein A, van der Linden FT, Voss A, Klaassen C,
364 Verdurin CM (2008) First outbreak of methicillin-resistant *Staphylococcus*
365 *aureus* ST398 in a Dutch hospital, June 2007. Eurosurveillance 13:pii=
366 8051.

367 8. Moodley A, Nielsen SS, Guardabassi L (2011) Effects of tetracycline and
368 zinc on selection of methicillin-resistant *Staphylococcus aureus* (MRSA)
369 sequence type 398 in pigs. Vet. Microbiol 152:420–423.

370 9. Bens CCPM, Voss A, Klaassen CHW (2006) Presence of a novel DNA
371 methylation enzyme in methicillin-resistant *Staphylococcus aureus*
372 isolates associated with pig farming leads to uninterpretable results in
373 standard pulsed-field gel electrophoresis analysis. J. Clin. Microbiol
374 44:1875–1876.

375 10. van Loo I, Huijsdens X, Tiemersma E, de Neeling A, van de Sande-
376 Bruinsma N, Beaujean D, Voss A, Kluytmans J (2007) Emergence of

377 methicillin-Resistant *Staphylococcus aureus* of animal origin in humans.
378 Emerg. Infect. Dis 13:1834–1839.

379 11. Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M (2005) Methicillin-
380 resistant *Staphylococcus aureus* in pig farming. Emerg. Infect. Dis
381 11:1965–1966.

382 12. Garcia-Graells C, Antoine J, Larsen J, Catry B, Skov R, Denis O (2012)
383 Livestock veterinarians at high risk of acquiring methicillin-resistant
384 *Staphylococcus aureus* ST398. Epidemiol. Infect 140:383–389.

385 13. Graveland H, Wagenaar JA, Bergs K, Heesterbeek H, Heederik D (2011)
386 Persistence of livestock associated MRSA CC398 in humans is
387 dependent on intensity of animal contact. PLoS One 6:e16830.

388 14. Köck R, Loth B, Köksal M, Schulte-Wülwer J, Harlizius J, Friedrich AW
389 (2012) Persistence of nasal colonization with livestock-associated
390 methicillin-resistant *Staphylococcus aureus* in pig farmers after holidays
391 from pig exposure. Appl. Environ. Microbiol 78:4046–4047.

392 15. Lozano C, Rezusta A, Gómez P, Gómez-Sanz E, Báez N, Martin-Saco G,
393 Zarazaga M, Torres C (2012) High prevalence of *spa* types associated
394 with the clonal lineage CC398 among tetracycline-resistant methicillin-
395 resistant *Staphylococcus aureus* strains in a Spanish hospital. J.
396 Antimicrob. Chemother 67:330–334.

397 16. Hallin M, De Mendonça R, Denis O, Lefort A, El Garch F, Butaye P,
398 Hermans K, Struelens MJ (2011) Diversity of accessory genome of

399 human and livestock-associated ST398 methicillin resistant
400 *Staphylococcus aureus* strains. Infect. Genet. Evol 11:290–299.

401 17. Witte W, Strommenger B, Stanek C, Cuny C (2007) Methicillin-resistant
402 *Staphylococcus aureus* ST398 in humans and animals, Central Europe.
403 Emerg. Infect. Dis 13:255–258.

404 18. Pan A, Battisti A, Zoncada A, Bernieri F, Boldini M, Franco A, Giorgi M,
405 Lurescia M, Lorenzotti S, Martinotti M, Monaco M, Pantosti A (2009)
406 Community-acquired methicillin-resistant *Staphylococcus aureus* ST398
407 infection, Italy. Emerg. Infect. Dis 15:845–847.

408 19. Ekkelenkamp MB, Sekkat M, Carpaij N, Troelstra A, Bonten MJM (2006)
409 Endocarditis due to meticillin-resistant *Staphylococcus aureus* originating
410 from pigs. Ned. Tijdschr. Geneeskd 150:2442–2447.

411 20. Armand-Lefevre L, Ruimy R, Andremont A (2005) Clonal Comparison of
412 *Staphylococcus aureus* Isolates from Healthy Pig Farmers, Human
413 Controls, and Pigs. Emerg. Infect. Dis 11:711–714.

414 21. Vindel A, Trincado P, Cuevas O, Ballesteros C, Bouza E, Cercenado E.
415 (2014) Molecular epidemiology of community-associated methicillin-
416 resistant *Staphylococcus aureus* in Spain: 2004-12. J. Antimicrob.
417 Chemother 69:2913–2919.

418 22. Camoez M, Sierra JM, Pujol M, Hornero A, Martin R, Domínguez MA.
419 (2013) Prevalence and molecular characterization of methicillin-resistant

Staphylococcus aureus ST398 resistant to tetracycline at a Spanish hospital over 12 years. PLoS One 8:e72828.

23. Clinical and Laboratory Standards Institute (CLSI): Performance Standards for Antimicrobial Susceptibility Testing: Twenty-second Informational Supplement. M100-S22. Wayne, PA, USA; 2012.

24. Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, Aires de Sousa M, Camou T, Cocuzza C, Corso A, Couto I, Dominguez A, Gniadkowski M, Goering R, Gomes A, Kikichi K, Marchese A, Mato R, Melter O, Oliveira D, Palacio R, Sá-Leao R, Sanches IS, Song JH, Tassios PT, Villari P (2000)Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb. Drug Resist 6:189–198.

25. van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, Fussing V, Green J, Feil E, Gerner-Smidt P, Brisse S, Struelens M (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13 Suppl 3:1-46.

26. Enright MC, Day NPJ, Davies CE, Peacock SJ, Spratt BG (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol 38:1008–1015.

441 27. Lina G, Boutite F, Tristan A, Bes M, Etienne J, Vandenesch F (2003)
442 Bacterial competition for human nasal cavity colonization: role of
443 Staphylococcal *agr* alleles. Appl. Environ. Microbiol 69:18–23.

444 28. Milheiriço C, Oliveira DC, de Lencastre H (2007) Update to the multiplex
445 PCR strategy for assignment of *mec* element types in *Staphylococcus*
446 *aureus*. Antimicrob. Agents Chemother 51:3374–3377.

447 29. Strommenger B, Braulke C, Heuck D, Schmidt C, Pasemann B, Nübel U,
448 Witte W (2008) *spa* Typing of *Staphylococcus aureus* as a frontline tool in
449 epidemiological typing. J. Clin. Microbiol 46:574–581.

450 30. Monecke S, Slickers P, Ehricht R (2008) Assignment of *Staphylococcus*
451 *aureus* isolates to clonal complexes based on microarray analysis and
452 pattern recognition. FEMS Immunol. Med. Microbiol 53:237–51.

453 31. Cavaco LM, Hasman H, Aarestrup FM (2011) Zinc resistance of
454 *Staphylococcus aureus* of animal origin is strongly associated with
455 methicillin resistance. Vet. Microbiol 50:344–348.

456 32. Kadlec K, Feßler AT, Hauschild T, Schwarz S (2012) Novel and
457 uncommon antimicrobial resistance genes in livestock-associated
458 methicillin-resistant *Staphylococcus aureus*. Clin. Microbiol. Infect
459 18:745–755.

460 33. Kadlec K, Ehricht R, Monecke S, Steinacker U, Kaspar H, Mankertz J,
461 Schwarz S (2009) Diversity of antimicrobial resistance pheno- and

462 genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from
463 diseased swine. J. Antimicrob. Chemother 64:1156–1164.

464 34. European Medicines Agency, European Surveillance of Veterinary
465 Antimicrobial Consuption (2013) Sales of veterinary antimicrobial agents
466 in 25 EU/EEA countries in 2011.(EMA/236501/2013)

467 35. Lozano C, Aspiroz C, Sáenz Y, Ruiz-García M, Royo-García G, Gómez-
468 Sanz E, Ruiz-Larrea F, Zarazaga M, Torres C (2012) Genetic
469 environment and location of the *lnu*(A) and *lnu*(B) genes in methicillin-
470 resistant *Staphylococcus aureus* and other staphylococci of animal and
471 human origin. J. Antimicrob. Chemother 67:2804–2808.

472 36. Stegger M, Liu CM, Larsen J, Soldanova K, Aziz M, Contente-Cuomo T,
473 Petersen A, Vandendriessche S, Jiménez JN, Mammina C, van Belkum
474 A, Salmenlinna S, Laurent F, Skov RL, Larsen AR, Andersen PS, Price
475 LB (2013) Rapid differentiation between livestock-associated and
476 livestock-independent *Staphylococcus aureus* CC398 clades. PLoS ONE
477 8:e79645.

478 37. Uhlemann AC, Porcella SF, Trivedi S, Sullivan SB, Hafer C, Kennedy AD,
479 Barbian KD, McCarthy AJ, Street C, Hirschberg DL, Lipkin WI, Lindsay
480 JA, DeLeo FR, Lowy FD (2012). Identification of a highly transmissible
481 animal-independent *Staphylococcus aureus* ST398 clone with distinct
482 genomic and cell adhesion properties. mBio 3:e00027-12.

483 38. McCarthy AJ, Witney AA, Gould KA, Moodley A, Guardabassi L, Voss A,
484 Denis O, Broens EM, Hinds J, Lindsay JA (2011). The distribution of

485 mobile genetic elements (MGEs) in MRSA CC398 is associated with both
486 host and country. Genome Biol. Evol 3:1164-1174.

487 39. Wellinder-Olsson C, Florén-Johansson K, Larsson L, Oberg S, Karisson
488 L, Ahrén C (2008) Infection with Panton-Valentine leukocidin-positive
489 methicillin-resistant *Staphylococcus aureus* t034. Emerg. Infect. Dis
490 14:1271–1272.

491 40. Huijsdens XW, Bosch T, van Santen-Verheuvel MG, Spalburg E, Pluister
492 GN, van Luit M, Heck ME, Haenen A, de Neeling AJ (2009) Molecular
493 characterization of PFGE non-typable methicillin-resistant *Staphylococcus*
494 *aureus* in The Netherlands, 2007. Euro Surveill 14:pii=19335.

495 41. Williamson DA, Bakker S, Coombs GW, Tan HL, Monecke S, Heffernan H
496 (2013) Emergence and molecular characterization of clonal complex 398
497 (CC398) methicillin-resistant *Staphylococcus aureus* (MRSA) in New
498 Zealand. J Antimicrob Chemother 69:1428-1430.

499 42. Fluit, AC (2012) Livestock-associated *Staphylococcus aureus*. Clin
500 Microbiol Infect 18:735-744.

501

Table 1. Characteristics of MRSA-ST398 isolated in southern Barcelona metropolitan area, Spain 2012-2013 (n=16)

PFGE	SCC*mec*	*agr*	*spa*	Resistance phenotype[a]	Resistance genotype	Virulence genes[b]	Biofilm genes[c]	MSCRAMM genes[d]	Patient[e]	No. Isolates
A	V	I	t011	TET	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	bbp, cflA, cflB, cna, fnbA	1	1
				TET, CIP	*tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	2;3	2
					tetK, tetM, czrC	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	5;10	2
				ERY, CLI, TOB, TET, CIP	*tetK, tetM, ermB, ermC, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB,*	9	1
				CLI, GEN, TOB, TET, CIP	*tetK, tetM, aacA/aphD, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	11	1
			t1451	ERY, CLI, TET, CIP	*tetK, tetM, ermC, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	4	1
			t5524	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	13	1
B	IV	I	t899	TET, CIP	*tetM*	*hlgA, hlgB, hlgC, hla, hld, scn, sak, chp*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	7	1
C	V	I	t011	TET	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	8	1
D	V	I	t011	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld,*	icaA, icaC, icaD	*cflA, cflB, can, fnbA, fnbB*	6	1
				ERY, CLI, TET, CIP	*tetK, ermC, czrC*	*hlgA, hlgB, hlgC, hla, hld, scn, sak, chp*	icaA, icaC, icaD	*cflA, cflB, cna, fnbA, fnbB*	12	1
E	V	I	t011	TET, CIP	*tetK, tetM, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	15	1
			t1451	ERY, CLI, TOB, TET, CIP	*tetK, tetM, ermC, aadD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*bbp, cflA, cflB, cna, fnbA, fnbB*	16	1
F	V	I	t011	ERY, CLI, GEN, TOB, TET	*tetK, tetM, ermC, aacA/aphD, czrC*	*hlgA, hlgB, hlgC, hla, hld*	icaA, icaC, icaD	*cflA, cflB, cna, fnbA, fnbB*	14	1
Total Nº of Isolates										16

[a]TET, tetracycline; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin.

[b] *hlgA,* gamma-hemolysin component A; *hglB,* gamma-hemolysin component B; *hglC*, gamma-hemolysin component C; *hla,* alpha-hemolysin*; hld,* delta-hemolysin*; scn,* staphylococcal complement inhibitor*; sak,* staphylokinase*; chp,* chemotaxis inhibitory protein*.*

[c] *icaA,* intercellular adhesion biofilm gene A; *icaC,* intercellular adhesion biofilm gene C; *icaD,* intercellular adhesion biofilm gene C.

[d] *bbp*, sialoprotein binding protein; *cflA,* clumping factor A; *cflB,* clumping factor B; *cna,* collagen binding protein; *fnbA,* fibronectin binding protein A; *fnbB,* fibronectin binding protein B

[e] Patient code

509 **Table 2 – Patients colonized or infected by MRSA-ST398 in southern Barcelona metropolitan area, Spain 2012-2013 (n=16)**

Patient	Gender ; Age (years)	Comorbidities[a]	Sample source[b]	Patient Status[c]	Previous MRSA-ST398 carriage	Persistence (months)[d]	Acquisition[e]
1	F ; 33	Obesity	Nares	C	No	-	CA
2	M ; 54	RA	Nares	C	No	-	CA
3	M ; 45	DM, HIV, HCV, RF	Nares	C	No	-	CA
4	M ; 72	DM, CD	SST	I	Yes	7	CA
5	M ; 58	DM, COPD, Cancer	SST	I	Yes	29	CA
6	F ; 59	HCV, HT	Nares	C	No	66	CA
7	F ; 54	Cancer	SST	I	No	-	HA
8	M ; 65	Obesity, DM, Cancer	Bone	I	Yes	1	CA
9	M ; 59	Cancer	Nares	C	No	20	CA
10	F ; 47	NA	Nares	C	No	-	CA
11	F ; 42	CD	SST	I	No	-	HA
12	F ; 67	DM, HBV, CD, Cancer	Nares	C	No	-	CA
13	M ; 81	CD, Cancer	Nares	C	No	5	CA
14	M ; 78	CD	Tracheotomy	C	No	-	HA
15	F ; 40	Cancer	Nares	C	No	-	CA
16	M ; 64	COPD	Blood	I	Yes	1	CA

510 [a] *RA, rheumatoid arthritis;* DM, diabetes mellitus; HIV, *human immunodeficiency virus; HCV, hepatitis C virus; RF, renal failure; CD, cardiovascular disease;* COPD,

511 chronic *obstructive pulmonary disease;* HT*, hepatic transplantation; NA, not available; HBV, hepatitis B virus*

512 [b] STT, skin and soft tissue

513 [c] C, colonization; I, Infection

514 [d] Hyphens stand for negative persistence

515 [e] CA, community-acquired; HA, hospital-acquired.

Figure S1 – Dendogram of *Apa*I-Pulsed-field Gel Electrophoresis (PFGE) representing the genetic relationship among 16 MRSA-ST398 isolates. Multilocus sequence typing (MLST), staphylococcal protein A (*spa*), staphylococcal cassette chromosome (SCC*mec*) and accessory gene regulator (*agr*) typing, year of isolation and patient data were shown at the right side of the figure. Lambda ladder marker was included in two lanes to normalize the PFGE patterns. The similarities among the fingerprints were calculated using the Jaccard coefficient (optimization, 0%; band position tolerance, 0.7%). The cut-off value to define the PFGE patterns was set as 80% similarity.

ORIGINAL ARTICLE BACTERIOLOGY

Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry

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Abstract

Early identification of methicillin-resistant *Staphylococcus aureus* (MRSA) dominant clones involved in infection and initiation of adequate infection control measures are essential to limit MRSA spread and understand MRSA population dynamics. In this study we evaluated the use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) for the automated discrimination of the major MRSA lineages (clonal complexes, CC) identified in our hospital during a 20-year period (1990–2009). A collection of 82 well-characterized MRSA isolates belonging to the four main CCs (CC5, CC8, CC22 and CC398) was split into a reference set (n = 36) and a validation set (n = 46) to generate pattern recognition models using the ClinProTools software for the identification of MALDI-TOF/MS biomarker peaks. The supervised neural network (SNN) model showed the best performance compared with two other models, with sensitivity and specificity values of 100% and 99.11%, respectively. Eleven peaks (m/z range: 3278–6592) with the highest separation power were identified and used to differentiate all four CCs. Validation of the SNN model using ClinProTools resulted in a positive predictive value (PPV) of 99.6%. The specific contribution of each peak to the model was used to generate subtyping reference signatures for automated subtyping using the BioTyper software, which successfully classified MRSA isolates into their corresponding CCs with a PPV of 98.9%. In conclusion, we find this novel automated MALDI-TOF/MS approach to be a promising, powerful and reliable tool for *S. aureus* typing.

Keywords: Clonal lineages, epidemiology, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, methicillin-resistant *Staphylococcus aureus*, typing

Original Submission: 6 August 2015; **Revised Submission:** 28 September 2015; **Accepted:** 5 October 2015

Editor: D. Raoult

Article published online: XXX

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important human pathogens causing nosocomial infections worldwide, and is represented by a limited number of genetic lineages [1].

Several molecular typing techniques, such as, pulsed-field gel electrophoresis, *S. aureus* protein A (*spa*) typing, multilocus sequence typing (MLST) or staphylococcal chromosome cassette *mec* typing are used to identify local MRSA-dominant clones and for epidemiological purposes [2,3]. Despite having a high discriminatory power, these molecular methods are time-consuming, expensive and require highly qualified personnel [4].

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) was recently

http://dx.doi.org/10.1016/j.cmi.2015.10.009

introduced into the clinical practice for bacterial identification [5–11], but the ability of this method to discriminate between different subspecies, identify distinct bacterial lineages, track outbreaks and find characteristics unique to certain strains has also been suggested, highlighting the potential of MALDI-TOF/MS as a very powerful epidemiological tool [7–9,12–18]. Nevertheless, all currently published MALDI-TOF/MS approaches require manual assessment of acquired spectra and highly trained personnel. Unfortunately, an automated protocol to detect different MRSA lineages has not yet been evaluated.

The present study aimed to analyse the potential of MALDI-TOF/MS to discriminate the major MRSA lineages circulating in our hospital using an automated approach.

Materials and methods

Bacterial isolates

This study included 95 MRSA clinical isolates recovered from single patients admitted to Hospital Universitari de Bellvitge (HUB) from 1990 to 2009. The isolates were representative of main MRSA epidemic clones (see Supplementary material, Table S1). All isolates were recovered from blood cultures except for strains belonging to the CC398 group (nasopharynx, n = 7; upper respiratory tract, n = 4; skin and soft tissue, n = 4; blood, n = 1 and central nervous system, n = 1). Hence, the only isolates recovered from non-invasive infections were 15 isolates of CC398. All isolates were characterized by pulsed-field gel electrophoresis, MLST, and staphylococcal chromosome cassette *mec, spa* and *agr* typing [3,19–22]. Allocation to specific clonal complexes (CCs) was based on the eBURST algorithm (http://eburst.mlst.net) [23]. The presence of genes encoding class S (*lukS-PV*) and class F (*lukF-PV*) proteins for Panton–Valentine leucocidin was studied by PCR in all isolates [24].

Sample preparation and MALDI-TOF/MS data acquisition

Bacterial cultures were grown overnight on Columbia sheep blood agar (Becton Dickinson GmbH, Heidelberg, Germany) at 37°C and subjected to ethanol–formic acid extraction as previously described [25].

MALDI-TOF/MS was conducted on a Microflex LT (Bruker Daltonik GmbH, Bremen, Germany) benchtop instrument controlled by the FlexControl software (version 3.0; Bruker Daltonics). Spectra were acquired in linear positive mode at a laser frequency of 20 Hz using the MALDI BioTyper (version 2.0; Bruker Daltonics) pre-processing standard method and the BioTyper main spectrum (MSP) identification standard method (mass range 2000–20 000 Da). The spectra were externally calibrated using the Bruker Daltonics Bacterial Test Standard.

One microlitre of each bacterial extract was spotted onto a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics) and air-dried at room temperature. For MALDI-TOF/MS analysis, each spotted sample was overlaid with 1 µL of a saturated matrix solution (α-cyano-4-hydroxy-cinnamic acid; Bruker Daltonics) in 50% acetonitrile–2.5% trifluoroacetic acid (Sigma-Aldrich Quimica SL, Madrid, Spain) and air-dried. The acquired spectrum for each spot was the sum spectrum accumulated from 250 measurements (5×50 laser shots on different locations according to a pre-defined lattice raster).

Selected bacterial isolates were included within a reference set (36 strains) or a validation set (46 strains). Selection was performed so as to include as much diversity as possible within both sets, prioritizing the reference set whenever an equitable distribution was not possible. Selection criteria were based on MLST variation or on *spa*-typing whenever MLST was too homogeneous. Bacterial extracts of the reference and validation sets were spotted onto a MALDI target plate in ten and three replicas, respectively. Spots for the reference set were measured three times, resulting in 30 mass spectra for each isolate, and those of the validation set were analysed twice, resulting in six mass spectra per isolate.

MALDI-TOF/MS data analysis

The resulting raw spectra for each isolate of the reference set were loaded into the ClinProTools software (version 2.2; Bruker Daltonics) and grouped into four different classes, each class containing the spectra profiles of isolates belonging to the same CC, and used to calculate the average spectra for each class (CC). Spectra were prepared by recalibration, baseline subtraction (Top Hat with a 10% minimal baseline width), peak selection and average peak list calculation ranging from 2000 to 10 000 mass to charge ratio values (m/z). Values of m/z from the average spectra of each CC were extracted and informative peaks were identified according to their statistical significance, as determined by the different statistical tests supported by ClinProTools (Bruker Daltonics): Anderson–Darling test, *t*-/analysis of variance (ANOVA) test and Wilcoxon/Kruskal–Wallis tests. Informative peaks were those showing a significant difference between the four CCs whether: the p value for the Anderson–Darling test was >0.05 and for the *t*-/ANOVA or Wilcoxon/Kruskal–Wallis test was $\leq$0.05 or if the p value for the Anderson–Darling test was $\leq$0.05 and for the Wilcoxon/Kruskal–Wallis test was $\leq$0.05 [26].

Classification models were generated using the genetic algorithm, supervised neural network (SNN), and QuickClassifier algorithms, to identify the algorithm providing the best recognition capability. For model preparation, null spectra exclusion and spectra grouping were enabled. Recalibration was performed with a 1000 parts per million maximal peak shift and

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30% match to calibrant peaks. Spectra that were not recalibrated were excluded. For model generation, the default settings were left unaltered. The recognition capability and cross validation values were calculated to demonstrate the reliability and accuracy of the model.

BioTyper MSPs representing each CC were created with the BioTyper MSP creation method using the following parameters: frequency threshold for spectra adjusting: 50; frequency threshold for score calculation: 5; maximal mass error of the raw spectrum: 2000; desired mass tolerance of the adjusted spectrum: 500; accepted mass tolerance of a peak: 800; parameter of the intensity correction function: 0.25. Subtyping MSPs for each CC were derived from the typing MSPs using the BioTyper subtyping MSP creation method with default parameters. Specific weights for each peak in the subtyping MSPs were set to 0% or replaced by the weight value calculated with the pattern generation models for informative peaks.

Results

Eighty-two MRSA clinical isolates representative of the four major MRSA CCs in our hospital were used to identify CC-specific biomarker peaks for MALDI-TOF/MS-based subtyping (see Supplementary material, Table S1). Isolates were divided in two sets—(i) a reference set of 36 isolates belonging to eight different sequence types (ST): ST146 (n = 5), ST125 (n = 5), ST228 (n = 5) from CC5, ST22 (n = 5) from CC22, ST398 (n = 5) from CC398, and ST8 (n = 5), ST247 (n = 4), ST1819 (n = 2) from CC8; and (ii) a validation set of 46 isolates belonging to six different ST and classified within the same CC as those of the reference set: ST146 (n = 5), ST125 (n = 5), ST228 (n = 5) of CC5, ST22 (n = 4) of CC22, ST398 (n = 12) of CC398 and ST8 (n = 15) of CC8.

An additional set of 13 isolates (see Supplementary material, Table S1) representing sporadic MRSA clones isolated in our hospital were also included: ST1 (n = 2) of CC1, ST30 (n = 2), ST36 (n = 1), ST714 (n = 1), ST1870 (n = 1) of CC30, ST45 (n = 1), ST1871 (n = 1) of CC45, ST72 (n = 3) of CC72 and ST88 (n = 1) of CC80.

TABLE 1. Performance of the pattern recognition models for the differentiation of the main methicillin-resistant *Staphylococcus aureus* clonal lineages generated by ClinProTools using the 36 strains from the reference set

Model	Max. peaks[a] (n)	Selected peaks[b] (n)	RC (%)	CV (%)
SNN	30	11	100	99.11
GA	30	7	100	94.53
QC	30	18	96.43	81.25

Abbreviations: CV, cross-validation; GA, genetic algorithm; QC, QuickClassifier; RC, recognition capability; SNN, supervised neural network.
[a]Maximum number of peaks for model generation.
[b]Number of peaks selected by the model.

Generation and validation of pattern recognition models

The acquired raw spectra for each isolate of the reference set were loaded into the ClinProTools software and grouped according to their corresponding CC. The average spectrum from each CC was calculated and used to generate pattern recognition models based on the genetic algorithm, SNN and QuickClassifier algorithms to identify an optimal set of peaks for the best class separation of the model generation spectra. The SNN algorithm yielded the model with the highest recognition capability and cross-validation values (100% and 99.11%, respectively; Table 1) and was therefore chosen to provide an optimal set of peaks for class discrimination. The model identified 11 peaks ranging from 3278 to 6592 m/z values as well as the specific contribution of each peak to the model (Table 2). Eight out of the 11 peaks showed low p values ($p \leq 0.05$) for the Anderson–Darling test, indicating the non-normal distribution

TABLE 2. ClinProTools peak statistics for the eleven peaks of interest[a]

Peak number	Mass	Weight	DAve	PTTA	PWKW	PAD	Ave1	Ave2	Ave3	Ave4
12	3278.56	0.01575683258213178	2.99	0.0000272	0.00934	<0.000001	2.64	2.51	2.7	5.51
16	3445.33	0.01537836986752119	30.57	0.000136	0.00116	0.164	15.93	27.23	46.5	37.35
20	3876.68	0.01770379511151569	4.73	0.0383	0.00137	<0.000001	1.16	5.89	5.88	2
21	3891.54	0.1795355741539599	4.37	0.0000535	0.000839	0.000109	4.95	0.89	1.08	0.58
32	4514.26	0.1323120474380986	3.16	0.0000274	0.00236	<0.000001	0.67	0.59	0.82	3.75
33	4540.27	0.01417947900114844	0.75	0.0000295	0.00116	0.645	1.43	1.7	1.95	2.19
38	4939.09	0.01460380606676501	1.46	0.0000295	0.00619	0.297	2.07	2.09	2.73	3.53
39	5004.15	0.1525008677385 17	19.07	0.0000624	0.00137	<0.000001	1.94	2.1	20.38	1.32
58	6481.8	0.14132700157690 95	2.21	<0.000001	0.00116	0.000238	2.65	0.49	2.61	2.7
60	6553.06	0.13481501061401 09	10.08	0.00000118	0.000449	0.033	8.34	2.15	6.52	12.24
62	6591.84	0.16774986054629 07	7.98	<0.000001	0.000503	<0.000001	2.65	10.6	2.62	3.36

Abbreviations: Mass: m/z value; Weight: relative contribution of each peak to the model; DAve: difference between the maximal and the minimal average peak area/intensity of all classes; PTTA: p value of t-/analysis of variance test; PWKW: p value of Wilcoxon/Kruskal–Wallis test (preferable for non-normally distributed data); PAD: p value of Anderson–Darling test, which gives information about normal distribution (p-value AD $\leq$0.05, non-normally distributed; p-value AD >0.05, normally distributed); Ave1, Ave2, Ave3 and Ave4, peak area/intensity average of class 1 (CC5), class 2 (CC8), class 3 (CC22) and class 4 (CC398), respectively.
[a]Peak number: correlative numbering of the peak in the average spectra.

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of these data (Table 2) and, hence the p value of the Wilcoxon/Kruskal–Wallis test was preferred over the p value of the *t*-/ANOVA test to consider them as informative peaks. The remaining three peaks showed p values for the Anderson–Darling test >0.05 (normally distributed); hence, the *t*-/ANOVA was considered instead of Wilcoxon/Kruskal–Wallis test (Table 2). The statistical analysis showed that the intensity differences of the individual peaks were statistically significant, suggesting their potential to discriminate among spectra from all four CCs.

Fig. 1 shows the best peaks whose presence or absence is specific for a given CC. For instance, the biomarker peak found at 3891 *m/z* was only present in the spectra of CC5 strains and was absent from all other spectra (Fig. 1). Mass spectra from non-CC5 isolates exhibited a peak at 3876 *m/z*. A biomarker peak found at 6592 *m/z* was unique to the spectra of CC8 isolates and peaks at 6481 and 6553 *m/z* were always absent in CC8 isolates but present in all other CCs. Specific peaks for CC22 and CC398 at 5004 and 4514 *m/z*, respectively, were also identified. Fig. 2 shows the pairwise two-dimensional distribution for the best separating peaks and supports their potential to differentiate isolates belonging to different clonal complexes. For instance, peaks at 3876 and 3891 *m/z* clearly discriminate isolates belonging to CC5, peaks at 6553 and 6592 *m/z* distinguish isolates from CC8 and peaks at 4514 and 5004 *m/z* categorize isolates from CC398 and CC22, respectively.

An external validation of the model using the 46 isolates of the validation set was implemented to further assess the performance of the model. Six independent spectra per strain were loaded into the ClinProTools software and classified according to the 11 biomarker peaks selected with the SNN algorithm. All but one replica were correctly classified into their corresponding CC, resulting in a positive predictive value (PPV) of 99.6% (Table 3).

Automated classification of spectra

In order to attempt automated subtyping of MRSA isolates to their corresponding CCs using the MALDI BioTyper software, spectra from each strain within the reference set were used to generate BioTyper MSP signatures representative of all four CCs that were subsequently incorporated to our local taxonomy database. CC-specific MSPs were then used to create associated subtyping MSPs that were manually edited by resetting the weights of all peaks to 0% except for those peaks identified in the SNN model, which were modified to incorporate the weight values provided by the model (Table 2).

Spectra from all 46 isolates of the validation set were then analysed with the MALDI BioTyper software against a local database that contained the subtyping MSPs. The best subtyping MSP match was recorded for each spectrum and, with the exception of three spectra from two CC5 isolates that were identified as either CC22 or CC398 (logscore values $\leq$2.4), all spectra were correctly classified (logscore values $\geq$2.6) resulting in a PPV of 98.9% (Table 3). In view of these results, an arbitrary cut-off logscore value of 2.6 was chosen to determine the correct subtyping of test spectra.

To test the performance of the subtyping MSPs when faced against isolates belonging to CCs other than those included in

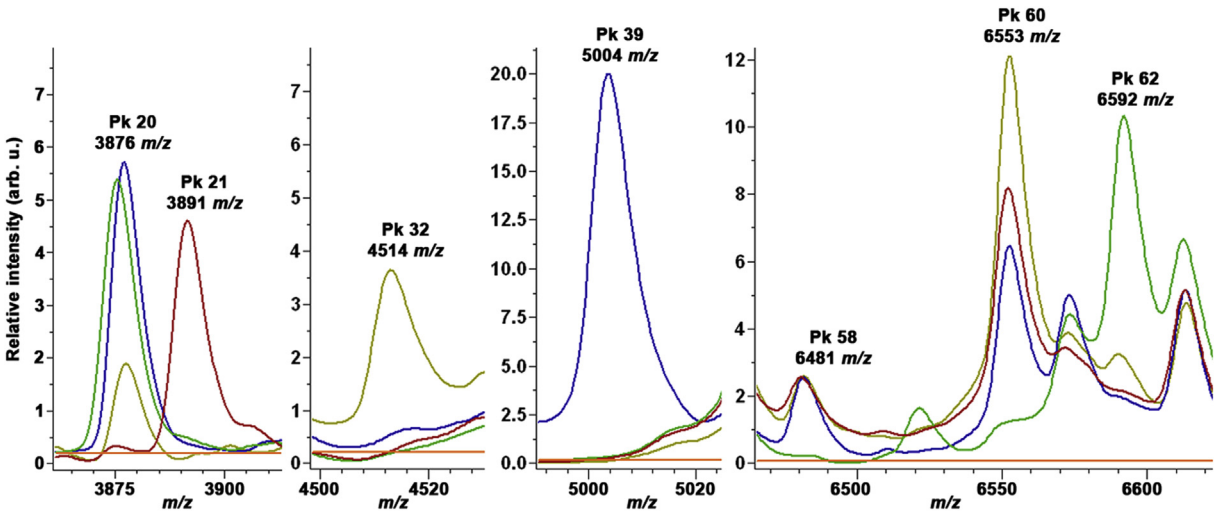

FIG. 1. Averaged spectra plots showing the presence or absence of the relevant peak biomarkers for matrix-assisted laser desorption ionization time-of-flight mass spectrometry discrimination of the four main methicillin-resistant *Staphylococcus aureus* clonal complexes in the supervised neural network model. CC5 (red), CC8 (green), CC22 (blue); CC398 (yellow). *x*-axis shows the mass per charge ratio values (*m/z*) and *y*-axis indicates the intensities of peaks expressed in arbitrary intensity units.

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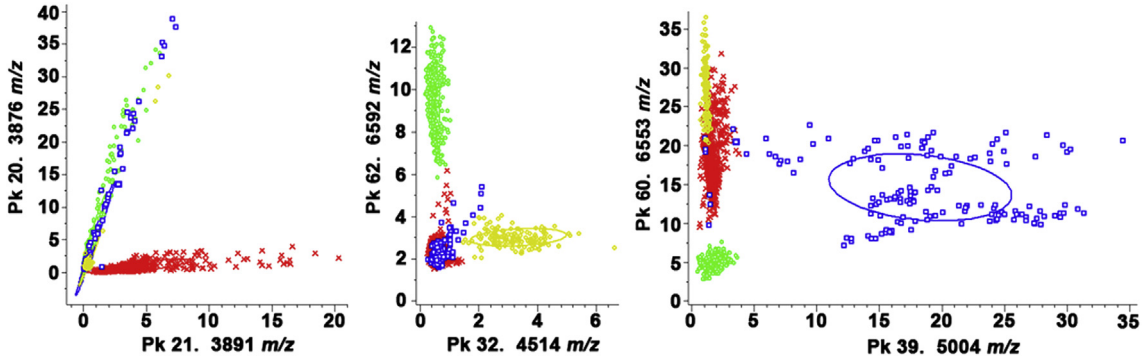

FIG. 2. Two-dimensional peak distribution diagrams displaying the paired distribution for the best separating peaks in the supervised neural network model; the ellipses represent the standard deviation of the class average of the peak areas/intensities. CC5 (red), CC8 (green), CC22 (blue); CC398 (yellow). The peak numbers and *m/z* values are indicated on the *x*- and *y*-axes, respectively.

the model, spectra from an additional set of 13 isolates representing sporadic MRSA clones (see Supplementary material, Table S1) were also analysed with the automated approach. As expected, the MALDI BioTyper aligned the peak profiles of sporadic isolates to the best matching subtyping reference spectrum and, therefore, attempted to classify them into one of the four CCs included in the database. The majority of the spectra were classified as belonging to either CC398 or CC22 with logscore values < 2.6. Five out of the 13 sporadic isolates (CC30 (n = 3), CC45 (n = 1) and CC80 (n = 1)), however, presented at least one spectrum with a logscore value $\geq$2.6 (Table 4), above the arbitrary confidence threshold.

Discussion

Previous studies introduced the use of MALDI-TOF/MS to discriminate MRSA clones or clonal lineages using different MALDI-TOF/MS approaches [1,27,28]. Wolters *et al.* described a preliminary MALDI-TOF-based MRSA typing scheme including 13 specific peaks that allowed the discrimination of five MRSA CCs (CC5, CC8, CC22, CC30 and CC45) [28]. However, this strategy had several limitations that made it difficult to implement in routine analysis, such as: no clear criteria for peak selection, tedious binary conversion and the requirement to generate hierarchical clusters for CC designation. More recently, Josten *et al.* evaluated the use of MALDI-TOF/MS to differentiate six *S. aureus* CCs (CC5, CC22, CC8, CC45, CC30 and CC1) by correlating peak shifts to point mutations, i.e. the genotypes of the strains [1]. Yet again, the methodology used required the close examination of spectra by expert personnel, making its implementation into routine diagnostic analysis not feasible.

In this study, we have evaluated for the first time a strategy that relies on a robust statistical analysis and the automated use of MALDI-TOF/MS to discriminate the major MRSA clonal lineages identified in our hospital. The ClinProTools statistical software was applied to generate and validate a pattern recognition SNN model that allowed the classification of MRSA epidemic clones predominant in our area with a sensitivity and specificity of 100% and 99.11%, respectively, providing a PPV of 99.6%. The SNN model identified 11 peaks with the highest separation power between all four CCs (3278, 3445, 3876, 3891, 4514, 4540, 4939, 5004, 6481, 6553 and 6591 *m/z*). Some

TABLE 3. External validation of the supervised neural network model using the ClinProTools and the MALDI BioTyper software

Software used	CC	No. of isolates	No. of spectra[a]	Spectra classification				Correct classified part of valid spectra (%)	PPV (%)
				CC5	CC8	CC22	CC398		
ClinProTools	5	15	90	90	0	0	0	100	99.6
	8	15	90	0	90	0	0	100	
	22	4	24	0	0	23	1	96	
	398	12	72	0	0	0	72	100	
MALDI BioTyper	5	15	90	87	0	2	1	96.7	98.9
	8	15	90	0	90	0	0	100	
	22	4	24	0	0	24	0	100	
	398	12	72	0	0	0	72	100	

Abbreviations: CC, clonal complex; PPV, positive predictive value.
[a]Each isolate was spotted three times and each spot was analysed twice, resulting in six mass spectra per isolate.

Please cite this article in press as: Camoez M, et al., Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.10.009

TABLE 4. Discriminatory power of the supervised neural network model performed in 13 isolates representing the minor methicillin-resistant *Staphylococcus aureus* clones circulating in the Hospital Universitari de Bellvitge using MALDI BioTyper software

Isolate	CC	ST	MALDI BioTyper classification (CC)[a]	Score[b]
1	1	1	398	2.597
2	1	1	398	2.498
3	**30**	**30**	**398**	**2.618**
4	30	714	398	2.517
5	**30**	**1870**	**398**	**2.609**
6	30	30	398	2.237
7	**30**	**36**	**398**	**2.613**
8	**45**	**45**	**398**	**2.606**
9	45	1871	398	2.544
10	72	72	398	2.288
11	72	72	398	2.371
12	72	72	22	2.435
13	**80**	**88**	**398**	**2.693**

Abbreviations: CC, clonal complex; ST, sequence type.
Isolates showing logscore values ≥2.6 are shown in bold type.
[a]Only the logscore with the highest value for all spectra originated from the same isolate is shown.
[b]Best score obtained for the subtyping classification of the six replicas.

of these peaks matched with those previously identified by Wolters *et al.* (3276, 3876, 4511, 5002 and 6591 *m/z*) and Josten *et al.* (3875, 3891, 4511, 5002, 6552 and 6592 *m/z*), so reinforcing the discriminatory value of our analysis [1,28]. Interestingly, those peaks showing a greater contribution to the model in our study (3876, 3891, 4514, 5004, 6553 and 6592 *m/z*) were already highlighted by Josten *et al.*, and peaks 3891, 5004 and 6592 have been identified as respective biomarkers for CC5, CC22 and CC8 in both studies. Of note, peak 4511 was identified as a biomarker for CC398 in our study while Josten *et al.* identified this peak as a biomarker also for CC30, CC45 and CC88, which were not included in our model because of the low number of strains in our collection achieving statistical significance. Close inspection of spectra from representative isolates belonging to such CCs identified the presence of peak 4511, confirming the results of Josten *et al.* [1].

The automated approach proved to be highly successful in the correct identification of isolates belonging to CCs included in the SNN model (PPV of 98.9%) and although it misidentified isolates from other CCs, it usually provided logscore values below our arbitrary threshold. Nevertheless, a few isolates belonging to CC30 (n = 3), CC45 (n = 1) and CC80 (n = 1) were incorrectly allocated to CC398 with logscore values above the threshold, probably due to the presence of the 4511 *m/z* peak that was identified in our model as a specific biomarker for CC398 but that is present in those CCs as well. We are aware that a recalculation of the model including representative strains from other CCs is needed to overcome this limitation. There were no relevant differences between the spectra from invasive versus non-invasive isolates of CC398, although the sample size was too small to perform a robust analysis. Differences between all isolates could only be attributed to their categorization into different CCs.

In summary, we have established a simple, automated and accurate methodology for the classification of MRSA clinical isolates into different CCs by MALDI-TOF/MS, which removes the need for batch or retrospective epidemiological analysis and allows inter-laboratory comparison. Further studies are needed to include additional CCs, but we find this novel automated MALDI-TOF/MS approach to be a promising, powerful and reliable tool for *S. aureus* typing that could easily be implemented in routine laboratories already using mass spectrometry.

Acknowledgements

The authors would like to thank Dr Dagmar Neimeyer and Dr Francesc Márquez, from Bruker Daltonics, for their excellent technical assistance. This study was funded by a grant from Fondo de Investigación Sanitaria (reference PI 10/01783), by the IMI (Innovation Medicine Initiatives) project COMBACTE (grant 115523) and by the Departament de Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (2014SGR06539). MC was supported by Plan Nacional de I+D+i 2008-2011 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Spanish Network for the Research in Infectious Diseases (grant number REIPI RD12/0015)—co-financed by European Development Regional Fund *A way to achieve Europe* ERDF. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. These data were partially presented at the 52nd Interscience Conference on Antimicrobial Agents and Chemotherapy San Francisco, USA, 2012 (communication no. D-1775).

Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.10.009.

Transparency declaration

The authors declare no conflicts of interest.

Please cite this article in press as: Camoez M, et al., Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.10.009

References

[1] Josten M, Reif M, Szekat C, Al-Sabti N, Roemer T, Sparbier K, et al. Analysis of the matrix-assisted laser desorption ionization-time of flight mass spectrum of *Staphylococcus aureus* identifies mutations that allow differentiation of the main clonal lineages. J Clin Microbiol 2013;51:1809–17.

[2] Struelens MJ, Hawkey PM, French GL, Witte W, Tacconelli E. Laboratory tools and strategies for methicillin-resistant *Staphylococcus aureus* screening, surveillance and typing: state of the art and unmet needs. Clin Microbiol Infect 2009;15:112–9.

[3] Milheiriço C, Oliveira DC, de Lencastre H. Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. Antimicrob Agents Chemother 2007;51:3374–7.

[4] Jackson KA, Edwards-Jones V, Sutton CW, Fox AJ. Optimisation of intact cell MALDI method for fingerprinting of methicillin-resistant *Staphylococcus aureus*. J Microbiol Methods 2005;62:273–84.

[5] Carbonnelle E, Beretti JL, Cottyn S, Quesne G, Berche P, Nassif X, et al. Rapid identification of Staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2007;45:2156–61.

[6] Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, Raoult D, et al. MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol 2010;5:1733–54.

[7] Boggs SR, Cazares LH, Drake R. Characterization of a *Staphylococcus aureus* USA300 protein signature using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Med Microbiol 2012;61:640–4.

[8] Ayyadurai S, Flaudrops C, Raoult D, Drancourt M. Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. BMC Microbiol 2010;10:285.

[9] Bernardo K, Pakulat N, Macht M, Krut O, Seifert H, Fleer S, et al. Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2002;2:747–53.

[10] Lynn EC, Chung MC, Tsai WC, Han CC. Identification of *Enterobacteriaceae* bacteria by direct matrix-assisted laser desorptiom/ionization mass spectrometric analysis of whole cells. Rapid Commun Mass Spectrom 1999;13:2022–7.

[11] Williamson YM, Moura H, Woolfitt AR, Pirkle JL, Barr JR, Carvalho Mda G. Differentiation of *Streptococcus pneumoniae* conjunctivitis outbreak isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 2008;74:5891–7.

[12] Dieckmann R, Helmuth R, Erhard M, Malorny B. Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 2008;74:7767–78.

[13] Edwards-jones V, Claydon MA, Evason DJ, Gordon DB. Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. J Med Microbiol 2000;49:295–300.

[14] Du Z, Yang R, Guo Z, Song Y, Wang J. Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 2002;74:5487–91.

[15] Majcherczyk PA, McKenna T, Moreillon P, Vaudaux P. The discriminatory power of MALDI-TOF mass spectrometry to differentiate between isogenic teicoplanin-susceptible and teicoplanin-resistant strains of methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett 2006;255:233–9.

[16] Lartigue M-F, Kostrzewa M, Salloum M, Haguenoer E, Héry-Arnaud G, Domelier AS, et al. Rapid detection of "highly virulent" Group B *Streptococcus* ST-17 and emerging ST-1 clones by MALDI-TOF mass spectrometry. J Microbiol Methods 2011;86:262–5.

[17] Lau AF, Wang H, Weingarten RA, Drake SK, Suffredini AF, Garfield MK, et al. A rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for single-plasmid tracking in an outbreak of carbapenem-resistant *Enterobacteriaceae*. J Clin Microbiol 2014;52:2804–12.

[18] Wang L, Han C, Sui W, Wang M, Lu X. MALDI-TOF MS applied to indirect carbapenemase detection: a validated procedure to clearly distinguish between carbapenemase-positive and carbapenemase-negative bacterial strains. Anal Bioanal Chem 2013;405:5259–66.

[19] Chung M, De Lencastre H, Matthews P, Tomasz A, Adamsson I, Aires de Sousa M, et al. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb Drug Resist 2000;6:189–98.

[20] Enright MC, Day NPJ, Davies CE, Peacock SJ, Spratt B. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol 2000;38:1008–15.

[21] Strommenger B, Braulke C, Heuck D, Schmidt C, Pasemann B, Nübel U, et al. *spa* typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. J Clin Microbiol 2008;46:574–81.

[22] 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.

[23] Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 2004;186:1518–30.

[24] Lina G, Pie Y, Godail-gamot F, Peter M. Involvement of Panton–Valentine leukocidin – producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis 1999;29:1128–32.

[25] Espinal P, Seifert H, Dijkshoorn L, Vila J, Roca I. Rapid and accurate identification of genomic species from the *Acinetobacter baumannii* (Ab) group by MALDI-TOF MS. Clin Microbiol Infect 2012;18:1097–103.

[26] Stephens MA. EDF statistics for goodness of fit and some comparisons. JASA 1974;69:730–7.

[27] Schlebusch S, Price GR, Hinds S, Nourse C, Schooneveldt JM, Tilse MH, et al. First outbreak of PVL-positive nonmultiresistant MRSA in a neonatal ICU in Australia: comparison of MALDI-TOF and SNP-plus-binary gene typing. Eur J Clin Microbiol Infect Dis 2010;29:1311–4.

[28] Wolters M, Rohde H, Maier T, Belmar-Campos C, Franke G, Scherpe S, et al. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. Int J Med Microbiol 2011;301:64–8.

Please cite this article in press as: Camoez M, et al., Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.10.009

Annex II

Additional scientific publications

Publications in international journals

1. Gasch O, Hornero A, Domínguez MA, Fernández A, Suárez C, Gómez S, Camoez M, Linares J, Ariza J, Pujol M. Methicillin-susceptible *Staphylococcus aureus* clone related to the early pandemic phage type 80/81 causing an outbreak among residents of three occupational centres in Barcelona, Spain. *Clin Microbiol Infect.* 2012. 18(7):662-7. (**IF: 5.768)**

2. Gasch O, Camoez M, Dominguez MA, Padilla B, Pintado V, Almirante B, Molina J, Lopez-Medrano F, Ruiz E, Martinez JA, Bereciartua E, Rodriguez-Lopez F, Fernandez-Mazarrasa C, Goenaga MA, Benito N, Rodriguez-Baño J, Espejo E, Pujol M; REIPI/GEIH Study Groups. Predictive factors for mortality in patients with methicillin-resistant *Staphylococcus aureus* bloodstream infection: impact on outcome of host, microorganism and therapy. *Clin Microbiol Infect*. 2013. 19(11):1049-57. (**IF: 5.768)**

3. Gasch O, Camoez M, Domínguez MA, Padilla B, Pintado V, Almirante B, Lepe JA, Lagarde M, Ruiz de Gopegui E, Martínez JA, Montejo M, Torre-Cisneros J, Arnáiz A, Goenaga MA, Benito N, Rodríguez-Baño J, Pujol M; REIPI/GEIH Study Groups. Predictive factors for early mortality among patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. *J Antimicrob Chemother*. 2013. 68(6):1423-30. (**IF: 5.313)**

4. Gasch O, Camoez M, Dominguez MA, Padilla B, Pintado V, Almirante B, Martín-Gandul C, López-Medrano F, de Gopegui ER, Ramón Blanco J, García-Pardo G, Calbo E, Horcajada JP, Granados A, Jover-Sáenz A, Dueñas C, Pujol M; REIPI/GEIM Study Groups. Lack of association between genotypes and haematogenous seeding infections in a large cohort of patients with methicillin-resistant *Staphylococcus aureus* bacteraemia from 21 Spanish hospitals. *Clin Microbiol Infect*. 2014. 20(4):361-7. (**IF: 5.768)**

5. Gasch O, Camoez M, Domínguez MA, Padilla B, Pintado V, Almirante B, Martín C, López-Medrano F, de Gopegui ER, Blanco JR, García-Pardo G, Calbo E, Montero M, Granados A, Jover A, Dueñas C, Pujol M; REIPI/GEIH study groups. Emergence of resistance to daptomycin in a cohort of patients with methicillin-resistant *Staphylococcus aureus* persistent bacteraemia treated with daptomycin. *J Antimicrob Chemother*. 2014. 69(2):568-71. (**IF: 5.313)**

6. Imaz A, Camoez M, Di Yacovo S, Gasch O, Dominguez MA, Vila A, Maso-Serra M, Pujol M, Podzamczer D. Prevalence of methicillin-resistant *Staphylococcus aureus* colonization in HIV-infected patients in Barcelona, Spain: a cross-sectional study. *BMC Infect Dis*. 2015. 26;15:243. **(IF: 2.61)**

ORIGINAL ARTICLE 10.1111/j.1469-0691.2011.03663.x

Methicillin-susceptible *Staphylococcus aureus* clone related to the early pandemic phage type 80/81 causing an outbreak among residents of three occupational centres in Barcelona, Spain

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Abstract

In the 1950s an unusually virulent and transmissible penicillin-resistant *Staphylococcus aureus* clone harbouring Panton–Valentine leukocidin (PVL) genes, known as phage type 80/81 and subsequently identified as multilocus sequence type (ST) 30, emerged and caused serious infections in hospitals and the community. We describe an outbreak of skin infections caused by a PVL-positive, methicillin-susceptible *S. aureus* (MSSA) strain of ST1472, related to phage type 80/81, in three associated occupational centres. After identification of the first patient an active case-finding strategy was initiated among the three centres. Epidemiological and clinical features were indistinguishable from outbreaks currently caused by community-acquired methicillin-resistant *S. aureus*. The *S. aureus* was cultured and identified from nasal swabs and skin lesions by conventional methods; PVL was detected using a PCR assay. Pulsed-field gel electrophoresis and DNA-array-based genotyping were applied to MSSA isolates. MSSA was identified in nasal swabs from 49 of 133 individuals (37%). A single pulsed-field gel electrophoresis pattern, belonging to ST1472 (CC30) and PVL positivity, were detected in 20 individuals, including eight of 18 skin cultures, i.e. 15% of the screened individuals were colonized by the epidemic strain. Nasal and cutaneous decontamination with 5% nasal mupirocin ointment and 2% aqueous chlorhexidine was implemented for all individuals. Patients with active skin infections were treated with a first-generation cephalosporin. General recommendations were made to prevent cross-transmission. No new cases were reported over the following 90 days.

Keywords: Methicillin-susceptible *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, outbreak, Panton–Valentine leukocidin, phage type 80/81

Original submission: 13 May 2011; **Revised submission:** 15 July 2011; **Accepted:** 24 August 2011

Editor: G. Lina

Clin Microbiol Infect

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Introduction

During the 1950s an unusually virulent and transmissible penicillin-resistant *Staphylococcus aureus* clone harbouring Panton–Valentine leukocidin (PVL) genes, belonging to phage type 80/81, emerged and caused serious skin and soft tissue infections in hospitals and the community. This strain was subsequently identified by multilocus sequence typing as ST30 [1], belonging to clonal complex (CC) 30. The pandemic phage type 80/81 disappeared during the 1960s as methicillin and its derivatives were used to treat penicillin-resistant staphylococcal infections. Current outbreaks of severe *S. aureus* skin and soft tissue infections detected worldwide tend to be caused by community-acquired methicillin-resistant strains (CA-MRSA) [2–4], although a few outbreaks caused by methicillin-susceptible *S. aureus* (MSSA) have recently been reported [5,6].

Panton–Valentine leukocidin was first described in 1894 [7] and was associated with skin and soft tissue infections in 1932 [8]. It is a leukocytolytic toxin comprising two different

proteins, encoded by the *lukS-PV* and *lukF-PV* genes. It has been related to recurrent severe skin infections, as well as to necrotizing pneumonia in previously healthy people without any contact with the health system [9]. Fewer than 5% of *S. aureus* strains in Europe harbour the PVL genes, although this percentage is much higher among strains causing cutaneous disease [10].

We describe an outbreak of skin and soft tissue infections in three related occupational centres, caused by a PVL-positive MSSA strain belonging to CC30 and related to the former pandemic phage type 80/81.

Materials and Methods

Setting

A private institution in the Barcelona area with three associated therapeutic centres for teenagers and young adults with behavioural disorders was studied. The three centres share staff, comprising psychologists, nurses, social workers, educators, teachers and monitors. For the purposes of therapy, residents usually move from one centre to another. Daily attendance at the three centres is around 130 boys and girls in a residential or outpatient setting.

Patients and methods

The first case was a social worker from one of the three centres who attended our emergency department in April 2010 with a skin abscess. She reported having furuncles over the past year and said there were other similar cases among staff and residents of all three centres, where she had been working for the last 2 years.

After identification of this case an active case-finding strategy was established in the three centres where an outbreak was suspected. A nasal swab was taken from all the residents and staff. Samples from skin ulcers were also taken from people with skin lesions.

Microbiology procedures

Swabs were plated onto coagulase–mannitol salt agar plates (BBL™ Coagulase Mannitol Agar; BD, Madrid, Spain) and inoculated into staphylococcal enrichment broth (BBL™ brain–heart infusion; BD, plus 7% NaCl). After incubation at 35°C for 24 h, broths were sub-cultured onto coagulase–mannitol salt agar plates. Colonies suggestive of *S. aureus* were identified by latex agglutination (Pastorex Staph-plus; Bio-Rad Laboratories, Madrid, Spain) and DNase production (DNase Test Agar; Biomérieux, Marcy l'Étoile, France). Antimicrobial susceptibility profiles were determined by the disk diffusion method with respect to the following antibiotics: penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampin, tetracycline, cotrimoxazole, chloramphenicol, vancomycin, teicoplanin, phosphomycin, fusidic acid, quinupristin/dalphopristin, linezolid and mupirocin. The criteria for antibiotic susceptibility were those recommended by the Clinical and Laboratory Standards Institute [11]. Genes encoding class S (*lukS-PV*) and class F (*lukF-PV*) proteins for PVL were amplified by PCR assay in all *S. aureus* isolates [9].

Pulsed-field gel electrophoresis (PFGE) was performed on all *S. aureus* isolates after *Sma*I restriction, following published methodology [12]. Restriction patterns of the PFGE were interpreted by visual inspection according to the criteria of van Belkum *et al.* [13] and further analysed with the FINGERPRINTING™ II software, version 3.0 (BioRad Laboratories, Inc., Madrid, Spain). The PFGE patterns were identified using a dendrogram generated by the unweighted-pair group method with arithmetic mean based on dice coefficients, where optimization and band position tolerance were set at 0.5% and 1.3%, respectively. A similarity coefficient of 80% was selected to define the patterns. [14] Multilocus sequence typing (MLST) and DNA microarray hybridization (StaphyType Kit, Alere Technologies Ltd, Jena, Germany; stripe version) were carried out in representative strains of the main clones, as described previously [15,16].

Interventions

To control further transmission, all residents and staff underwent nasal and skin decontamination during the same week, involving a daily shower (including hair) and using 2% aqueous chlorhexidine for 1 week and a nasal ointment with 5% mupirocin in both nostrils (three times a day for 5 days). During that week, they were all given general recommendations regarding increased hand washing, daily changing and washing of towels, bedclothes and underwear in water at a temperature of at least 60°C, and instructions emphasizing the importance of not sharing personal items. Primary-care physicians were informed and passive surveillance (requesting the reporting of relapsing skin infections) was performed. The local department of health was also informed about the investigation into the outbreak.

Results

Cultures from nasal swabs and active skin lesions were taken from all the residents and staff of the three centres. Overall, 133 individuals (95 residents and 38 members of staff) aged 14–55 years were screened (57% males and 43% females). Among the residents, 22 (17%) came from other countries

(nine Colombia, three Romania, two Peru, and one each from Nepal, Dominican Republic, Chile, Panama, Brazil, Mexico, Argentina and Bolivia). None of those screened had any previous diseases of note, except for one patient with chronic asthma, another with psoriasis and another with HIV infection (acquired by vertical transmission). At the time of the screening or within the previous 2 years, 34 (26%) of them reported skin lesions, which were described as ulcers, furuncles, cutaneous abscesses or folliculitis. Most of them had received systemic treatment with beta-lactams or cotrimoxazole and/or topical treatment with mupirocin or fusidic acid.

Cultures yielded MSSA in 49 of the 133 nasal swabs (37%) and in eight skin ulcers from the nine patients with cutaneous lesions at the time of screening. The OR for *S. aureus*-colonized versus non-colonized for skin and soft tissue infection during the outbreak period was 14.9 (95% CI 5.3–41.9). Isolates were susceptible to all of the antibiotics tested except for penicillin. MRSA was not detected in any sample. The PVL test was positive in MSSA isolates from 20 individuals, including all isolates from skin ulcers (n = 8). Therefore, 20 (15%) of the 133 screened patients were colonized by a PVL-positive MSSA (Table 1); OR (CC30 vs non-CC30 *S. aureus*) for skin and soft tissue infection during the outbreak period: 3.0 (95% CI 0.9–9.9).

Among PVL-positive MSSA strains isolated in both nasal and skin lesions, a single PFGE pattern was observed (pattern A). Seven PFGE restriction patterns (patterns B to H) were found among the 27 PVL-negative MSSA isolates (Fig. 1). Two patients were colonized by a PVL-negative variant of PFGE type A. The PFGE patterns C and B clustered 14 and eight patients, respectively. The unweighted-pair group method with arithmetic mean analysis placed the PFGE profiles A and C in the same cluster with similarity higher than 80% (see Fig. 1).

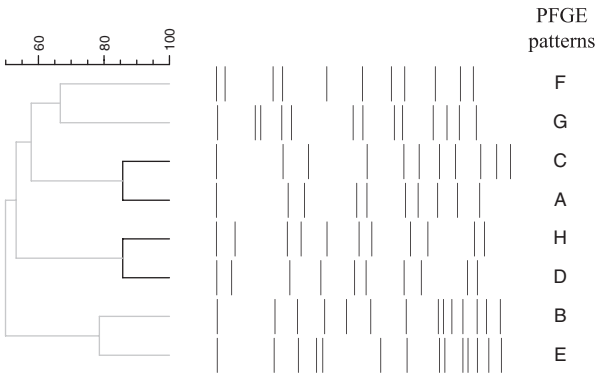

FIG. 1. Pulsed-field gel electrophoresis (PFGE) of *Sma*I macrorestriction fragments of methicillin-susceptible *S. aureus* isolates. The cut-off value for designing genotypes was set at 80%. For dendrogram construction tolerance was set at 1.25% and optimization at 0.5%.

The PVL-positive epidemic strain belonged to *agr* group III. Multilocus sequence typing was performed on four strains of PFGE type A (PVL-positive), two strains of PFGE type B and two strains of PFGE type C. The epidemic strains (PVL-positive PFGE type A strains) belonged to ST1472, a single locus variant of ST30, defined by alleles *arcc* 2, *aroe* 2, *glpf* 2, *gmk* 2, *pta* 6, *tpi* 3 and *yqi* 162. Strains of major PVL-negative PFGE patterns C and B belonged to ST30 and ST45, respectively.

TABLE 1. Demographic characteristics and treatment of patients with previous skin lesions and/or colonized by PVL producing *Staphylococcus aureus* (CC30)

Patient	Gender/Age	Role/Center	Nationality	Previous skin lesions	Sample yielding CC30 MSSA	Treatment
1	F/16	User/C	Spain	No	NS	NA
2	F/16	User/B	Spain	Yes	Both	Fusidic acid
3	M/16	User/A	Spain	No	NS	NA
4	M/21	Worker/C	Spain	Yes	NS	None
5	M/16	User/B	Spain	Yes	NS	NA
6	M/18	User/c	Perú	Yes	Both	Mupirocine
7	M/14	User/c	Spain	Yes	NS	Amoxicillin+ fusidic acid
8	M/16	User/c	Spain	Yes	SL	Cotrimoxazole
9(*)	F/39	Worker/ABC	Spain	Yes	SL	Amoxicillin+ fusidic acid
10	M/25	Worker/B	Spain	No	NS	NA
11	F/15	User/A	Perú	No	NS	NA
12	M/49	Worker/B	Spain	No	NS	NA
13	F/16	User/C	Spain	Yes	SL	Mupirocine
14	F/16	User/B	Spain	No	NS	NA
15	M/15	User/A	Spain	Yes	Both	Cloxacilin
16	F/17	User/A	Bolivia	Yes	NS	NA
17	M/18	User/B	Spain	Yes	NS	Fusidic acid
18	F/16	User/B	Spain	Yes	Both	Cotrimoxazole
19	F/28	Worker/B	Spain	No	NS	NA
20	F/17	User/A	Colombia	Yes	Both	NA

(*)First case. M Male, F Female. NA Not aported. NS Nasal Swap. SL Skin lesion

The DNA microarray analysis was performed on two separate PVL-positive PFGE type A strains which showed identical hybridization patterns. The epidemic strain showed a single positive hybridization on gene *blaZ* among genes encoding different antibiotic resistance markers. The epidemic strain carried the enterotoxin gene cluster (*egc*) comprising *selo*, *selm*, *sei*, *seln*, *seg* and *selu* genes. There were also positive hybridization signals for the *lukS/F*-PVL, *hlgA* (component A of γ-haemolysin), *hla* (α-haemolysin), *hlb* (β-haemolysin), *hld* (δ-haemolysin) and *sak* (staphylokinase) genes. The capsule was type 8. Genes for exfoliative toxins A, B and D were negative, as was the gene coding for the toxic shock syndrome toxin.

The microarray includes alleles coding for staphylococcal superantigen-like proteins (*set* genes) from several sequenced strains (MW0345, COL, Mu50, MW0382 and MRSA252). The epidemic strain tested here harboured a complex of *set* genes analogous to the *set* alleles found in strain MRSA252. Strain MRSA252 belongs to ST36 (alleles *arcc* 2, *aroe* 2, *glpf* 2, *gmk* 2, *pta* 3, *tpi* 3 and *yqi* 2), a single locus variant of ST30.

Patients with active skin lesions were treated for 2 weeks with a first-generation cephalosporin. After treatment the decontamination strategy was initiated for all staff and residents, with no new cases of skin infections appearing over the following 90 days.

Discussion

Our study is a comprehensive description of an outbreak of skin and soft tissue infections caused by a PVL-positive MSSA strain belonging to CC30 and related to the former pandemic phage type 80/81. Some aspects of our experience require more detailed comment.

From an epidemiological and clinical perspective the outbreak affected a closed community of teenagers and young adults. This age group has close contact and share activities and personal items that facilitate the transmission of an epidemic strain. One noteworthy finding was the high estimated attack rate of 26%, which illustrates a great ability of this strain to disseminate, as had been reported for MSSA phage type 80/81 in the early 1950s. Of note, the OR of CC30-colonized patients (compared with those patients colonized by other clones of *S. aureus*) for SSTI was three. However, it must be pointed out that it might be underestimated by the effect of treatment received by patients with lesions before the detection and study of the outbreak, which might achieve in some cases a persistent decontamination.

Although CA-MRSA is still infrequent in Spain it is being increasingly reported. The clinical and epidemiological features of the outbreak described here were indistinguishable from those reported for CA-MRSA outbreaks [17], and in fact the latter was our initial suspicion after seeing the first case. The skin lesions reported by residents and staff were predominantly severe and relapsing furunculosis, similar to those caused by CA-MRSA harbouring PVL in community settings such as football teams or schools.

Given the lack of specific recommendations for preventing epidemic MSSA infections, and in accordance with previous experience with MRSA infections [18,19], we advised the three centres of the need to perform a universal and active decontamination with mupirocin ointment and aqueous chlorhexidine for all staff and residents, irrespective of their carrier status for the epidemic strain. A number of general hygiene measures were also recommended to avoid transmission. As a result, no other case of MSSA infection was observed over the following 3 months.

The strain cultures in the furuncle from the first patient were identified as the epidemic MSSA strain, The multilocus sequence typing analysis revealed that it belonged to the CC30 lineage (ST1472) and carried a set of alleles identical to those of reference strain MRSA252, which also belongs to CC30 (ST36). The founder of CC30 is ST30, the same as that of the former pandemic phage type 80/81. As in our experience, phage type 80/81 was a highly transmissible strain that caused outbreaks of severe skin disease during the 1950s [1,20]. It was shown to be very invasive and one study reported that a third of nasal carriers of phage type 80/81 who were admitted to hospital developed septicaemia, compared with <5% of those colonized by other strains [21]. The isolation of this strain subsequently decreased, which could be related to the introduction of penicillinase-resistant β-lactams in the 1960s.

The MSSA strain described here had the most prevalent susceptibility pattern in Spain (resistance to penicillin, susceptibility to other tested antibiotics) [22], and had no other SCC*mec*-associated genes, as this has been screened with the DNA microarray hybridization. This differs from other reported clusters caused by multi-resistant MSSA strains, in which *mecA* remnants were found. These reports [6,23,24] concluded that their findings were the result of an *S. aureus* strain that had previously lost the *mecA* gen.

A regional distribution of *S. aureus* clones causing infections has been observed [25]. For instance, whereas most PVL *S. aureus*-related infections in the USA have been caused by ST8 USA300 CA-MRSA strains [20,25], the most prevalent strains in Europe belong to ST8, ST80, ST30 and 121 [25–28]. Hence, in contrast to what is seen in the USA, most of the infections caused by PVL-producing strains in Europe are MSSA. However, this scenario is probably over-

looked because no routine test is usually performed to detect PVL expression [29–31].

In summary, the paper describes an outbreak caused by a PVL-positive MSSA strain related to the former pandemic phage type 80/81. All the characteristics were indistinguishable from those of CA-MRSA outbreaks. The outbreak was controlled after active nasal and cutaneous decontamination.

Funding

This work was supported by a grant from Fondo de Investigación Sanitaria (PI07/0944) and by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III—co-financed by European Development Regional Fund 'A way to achieve Europe' ERDF, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008).

Transparency Declaration

No conflicts of interest.

References

1. Robinson DA, Kearns AM, Holmes A *et al.* Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired methicillin-resistant clone. *Lancet* 2005; 365: 1256–1258.
2. Kazakova SV, Hageman JC, Matava M *et al.* A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *N Engl J Med* 2005; 352: 468–475.
3. Baggett HC, Hennessy TW, Rudolph K *et al.* Community-onset methicillin-resistant *Staphylococcus aureus* associated with antibiotic use and the cytotoxin Panton-Valentine leukocidin during a furunculosis outbreak in rural Alaska. *J Infect Dis* 2004; 189: 1565–1573.
4. Begier EM, Frenette K, Barrett NL *et al.* A high-morbidity outbreak of methicillin-resistant *Staphylococcus aureus* among players on a college football team, facilitated by cosmetic body shaving and turf burns. *Clin Infect Dis* 2004; 39: 1446–1453.
5. Wiese-Posselt M, Heuck D, Draeger A *et al.* Successful termination of a furunculosis outbreak due to lukS-lukF-positive, methicillin-susceptible *Staphylococcus aureus* in a German village by stringent decolonization, 2002–2005. *Clin Infect Dis* 2007; 44: e88–e95.
6. Lindqvist M, Isaksson B, Samuelsson A, Nilsson LE, Hallgren A. A clonal outbreak of methicillin-susceptible *Staphylococcus aureus* with concomitant resistance to erythromycin, clindamycin and tobramycin in a Swedish county. *Scand J Infect Dis* 2009; 41: 324–333.
7. Van de Velde H. Etude sur le mécanisme de la virulence du Staphylocoque pyogène. *La Cellule* 1894; 10: 401–410.
8. Panton PN, Valentine FCO. Staphylococcal toxin. *Lancet* 1932; 219: 506–508.
9. Lina G, Piemont Y, Godail-Gamot F *et al.* Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 1999; 29: 1128–1132.
10. Masiuk H, Kopron K, Grumann D *et al.* Association of recurrent furunculosis with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus*. *J Clin Microbiol* 2010; 48: 1527–1535.
11. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; 20th Informational Supplement. CLSI document M100-S20*. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute, 2010.
12. Chung M, De Lencastre H, Matthews P *et al.* Molecular typing of methicillin-resistant *Staphylococcus aureus* (MRSA) by pulsed field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist*, 2000; 6: 189–197.
13. van Belkum A, Tassios PT, Dijkshoorn L *et al.* Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin Microbiol Infect* 2007; 13 (Suppl 3): 1–46.
14. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol* 2003; 41: 5113–5120.
15. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; 38: 1008–1015.
16. Monecke S, Ehricht R. Rapid genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates using miniaturised oligonucleotide arrays. *Clin Microbiol Infect* 2005; 11: 825–833.
17. Manzur A, Dominguez AM, Pujol M *et al.* Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. *Clin Microbiol Infect* 2008; 14: 377–380.
18. Pujol M, Pena C, Pallares R *et al.* Nosocomial *Staphylococcus aureus* bacteremia among nasal carriers of methicillin-resistant and methicillin-susceptible strains. *Am J Med* 1996; 100: 509–516.
19. Calfee DP, Salgado CD, Classen D *et al.* Strategies to prevent transmission of methicillin-resistant *Staphylococcus aureus* in acute care hospitals. *Infect Control Hosp Epidemiol* 2008; 29 (Suppl 1): S62–S80.
20. Monecke S, Berger-Bachi B, Coombs G *et al.* Comparative genomics and DNA array-based genotyping of pandemic *Staphylococcus aureus* strains encoding Panton-Valentine leukocidin. *Clin Microbiol Infect* 2007; 13: 236–249.
21. Williams RE, Jevons MP, Shooter RA *et al.* Nasal staphylococci and sepsis in hospital patients. *Br Med J* 1959; 2: 658–662.
22. Cuevas O, Cercenado E, Goyanes MJ *et al.* [*Staphylococcus* spp. in Spain: present situation and evolution of antimicrobial resistance (1986–2006)]. *Enferm Infecc Microbiol Clin* 2008; 26: 269–277.
23. Fontanilla JM, Kirkland KB, Talbot EA *et al.* Outbreak of skin infections in college football team members due to an unusual strain of community-acquired methicillin-susceptible *Staphylococcus aureus*. *J Clin Microbiol* 2010; 48: 609–611.
24. Donnio PY, Fevrier F, Bifani P *et al.* Molecular and epidemiological evidence for spread of multiresistant methicillin-susceptible *Staphylococcus aureus* strains in hospitals. *Antimicrob Agents Chemother* 2007; 51: 4342–4350.
25. Tristan A, Bes M, Meugnier H *et al.* Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. *Emerg Infect Dis* 2007; 13: 594–600.
26. Daskalaki M, Rojo P, Marin-Ferrer M, Barrios M, Otero JR, Chaves F. Panton-Valentine leukocidin-positive *Staphylococcus aureus* skin and soft tissue infections among children in an emergency department in Madrid, Spain. *Clin Microbiol Infect* 2010; 16: 74–77.
27. Monecke S, Slickers P, Ellington MJ, Kearns AM, Ehricht R. High diversity of Panton-Valentine leukocidin-positive, methicillin-susceptible isolates of *Staphylococcus aureus* and implications for the evolution of community-associated methicillin-resistant *S. aureus*. *Clin Microbiol Infect* 2007; 13: 1157–1164.
28. Rasigade JP, Laurent F, Lina G *et al.* Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible

Staphylococcus aureus, 1981–2007. *J Infect Dis* 2010; 201: 1589–1597.
29. Shallcross LJ, Williams K, Hopkins S, Aldridge RW, Johnson AM, Hayward AC. Panton-Valentine leukocidin associated staphylococcal disease: a cross sectional study at a London Hospital, England. *Clin Microbiol Infect* 2010; 16: 1644–1648.
30. Robert J, Etienne J, Bertrand X. Methicillin-resistant *Staphylococcus aureus* producing Panton-Valentine leukocidin in a retrospective case series from 12 French hospital laboratories, 2000–2003. *Clin Microbiol Infect* 2005; 11: 585–587.
31. Gillet Y, Issartel B, Vanhems P *et al.* Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* 2002; 359: 753–759.

ORIGINAL ARTICLE 10.1111/1469-0691.12108

Predictive factors for mortality in patients with methicillin-resistant *Staphylococcus aureus* bloodstream infection: impact on outcome of host, microorganism and therapy

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Abstract

Mortality related to methicillin-resistant *Staphylococcus aureus* (MRSA) bloodstream infection (BSI) remains high, despite changes in the epidemiology. To analyze the current predictive factors for mortality we conducted a prospective study in a large cohort of patients with MRSA-BSI from 21 Spanish hospitals. Epidemiology, clinical data, therapy and outcome were recorded. All MRSA strains were analysed, including susceptibility to antibiotics and molecular characterization. Vancomycin MICs (V-MIC) were tested by the *E*-test and microdilution methods. Time until death was the dependent variable in a Cox regression analysis. Overall, 579 episodes were included. Acquisition was nosocomial in 59% and vascular catheter was the most frequent source (38%). A dominant PFGE genotype was found in 368 (67%) isolates, which belonged to Clonal Complex (CC)5 and carried SCCmecIV and *agr*2. Microdilution V-MIC50 and V-MIC90 were 0.7 and 1.0 mg/L, respectively. Initial therapy was appropriate in 66% of episodes. Overall mortality was observed in 179 (32%) episodes. The Cox-regression analysis identified age >70 years (HR 1.88), previous fatal disease (HR 2.16), Pitt score >1 (HR 3.45), high-risk source (HR 1.85) and inappropriate initial treatment (HR 1.39) as independent predictive factors for mortality. CC5 and CC22 (HR 0.52 and 0.45) were associated with significantly lower mortality rates than CC8. V-MIC $\geq$ 1.5 did not have a significant impact on mortality, regardless of the method used to assess it.

Keywords: Mortality, MRSA bacteraemia, MRSA bloodstream infection, *Staphylococcus aureus*, vancomycin MIC
Original Submission: 31 August 2012; **Revised Submission:** 3 November 2012; **Accepted:** 18 November 2012
Editor: G. Lina

Clin Microbiol Infect

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) bloodstream infection (BSI) has been a cause of concern in healthcare systems around the world in recent decades, due to an increase in its incidence and undesirable related outcomes [1]. Despite the recent epidemiological changes, MRSA clonal replacement, clinical use of new antibiotics and improvement in supportive therapies, mortality related to MRSA-BSI remains close to 30% [2].

A variety of factors have been associated with worse outcomes among MRSA-BSI patients, including host characteristics, bacterial genetic background and therapeutic management [3]. At present, however, no predictors for mortality have been definitively established, because only a few recent large prospective multicentre studies have analysed all these factors together.

Vancomycin, which is the standard therapy for MRSA-BSI, has recently been the focus of attention. Its suboptimal *in vitro* killing activity for *S. aureus* compared with betalactams [4] and the observation of MIC creep in many hospitals around the world [5] have raised questions about its suitability for use against MRSA invasive infections [6,7]. Special concern has been raised during the last decade regarding the impact on mortality of MRSA strains with reduced vancomycin susceptibility [3,7–9].

This study aimed to assess the prognostic factors for mortality among MRSA-BSI patients, taking into account the current epidemiological data and carefully examining the clinical impact of vancomycin susceptibility on mortality.

Methods

Study period and patients

The study was conducted from June 2008 to December 2009 at 21 Spanish hospitals. Four centres had <500 beds, nine had 500–1000 beds and eight had >1000 beds. An infectious disease specialist prospectively followed adult patients (>16 years old) with MRSA blood cultures previously detected at the Microbiology Laboratory, and excluded the cases that did not meet the inclusion criteria, such as lack of signs and symptoms consistent with sepsis. A standardized protocol with demographic and clinical information, including age, sex, co-morbid conditions, source and acquisition, diagnostic explorations, antibiotic treatment, follow-up and outcome was followed. Strains were sent to a central laboratory for further analysis.

Study design

All the episodes included in the study were used to assess the prognostic factors for mortality, which was measured 30 days after the first blood culture. Two different models that only differed in the method used to assess MRSA vancomycin susceptibility (*E*-test or microdilution) were used. MICs were stratified as follows: <1.5 and $\geq$ 1.5 μg/mL. Time until death was the dependent variable in a Cox regression analysis and was censored 30 days after the end of therapy or end of follow-up. The prognostic factors for non-appropriate initial antibiotic therapy were analysed in a logistic regression model. Patients who received appropriate initial therapy were included in an adjusted multivariate analysis designed to assess the existence of differences in mortality related to the specific antibiotic administered.

Definitions

Methicillin-resistant *Staphylococcus aureus*-BSI is defined as the presence of at least one positive blood culture for MRSA in a blood sample from a patient with clinical findings consistent with infection. Co-morbidity was measured by the Charlson score, which stratifies the associated diseases into an ordinal scale. Patients were classified into three categories on the McCabe scale according to their prognosis of survival before the MRSA-BSI: non-fatal if death was expected within a period longer than 5 years; ultimately fatal if death was expected between 1 and 5 years; and rapidly fatal if it was expected in the following year. Severity of disease was assessed by the Pitt score. Three acquisition categories were considered according to Friedman criteria: (i) nosocomial-BSI if the episode was diagnosed at least 48 hours after the hospital admission, either to the ICU or to a conventional hospital ward (non-ICU), and if there were no signs or symptoms of infection at admission; (ii) healthcare related-bacteraemia if the patient had had contact with the healthcare system within the previous 3 months; and (iii) community-acquired otherwise. Source of the BSI was defined according to the CDC criteria [10]. BSI of unknown source (primary) was defined when its origin was uncertain after careful examination of the clinical and microbiological data. It was considered high risk if the source was lower respiratory tract, endocarditis or unknown [3]. Distant extension was diagnosed in the presence of at least one distant infection secondary to blood spread seeding. Persistence was defined as growth of MRSA in blood cultures after more than 48 h of appropriate antibiotic therapy. The initial antibiotic was defined as the antibiotics administered in the first 48 h after BSI onset, regardless of the microbiological information. Definitive treatment was considered as the antimicrobials administered after conducting microbiological sensitivity tests. Antibiotic treatment was considered appropriate if the strain was susceptible to at least one of the administered antibiotics, with the exception of aminoglycosides, which were considered inappropriate, regardless of the sensitivity tests. Source eradication was considered if the catheter or foreign body was removed, or if a surgical intervention or drainage of BSI source was performed.

Susceptibility testing and molecular epidemiology of MRSA isolates

Each hospital identified the strain and performed preliminary susceptibility tests. Isolates were sent to a central reference laboratory. All *S. aureus* were identified by latex agglutination (Pastorex Staph-plus, Bio-Rad Laboratories, Madrid, Spain) and DNase production (DNas *E*-test Agar, BioMérieux, Marcy l'Étoile, France). Antimicrobial susceptibility of all MRSA isolates was tested by the disk-diffusion method according to the Clinical Laboratory Standard Institute (CLSI) guidelines [11]. The antimicrobial agents tested were penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, gentamicin, tobramycin,

ciprofloxacin, rifampicin, trimethoprim-sulphamethoxazole, tetracycline, fosfomycin, vancomycin, teicoplanin, chloramphenicol, daptomycin and linezolid. Minimum inhibitory concentration (MIC) was determined by the microdilution method in accordance with CLSI criteria by using commercial panels (ESTEN 2009, Sensititre, Izasa, Barcelona, Spain) read visually. Vancomycin MICs were also studied by *E*-test (BioMérieux) on Mueller-Hinton agar, using a turbidity of 0.5 on the McFarland scale.

Pulsed-field gel electrophoresis (PFGE) was performed after SmaI restriction of chromosomal DNA[12]. Restriction patterns were interpreted in accordance with criteria published elsewhere [13]. To define PFGE types the FINGERPRINTING II software, version 3.0 (BioRad Laboratories, Inc., Madrid, Spain) was applied.

A dendrogram was generated by the unweighted-pair group method with arithmetic mean based on Dice coefficients. To define PFGE types the similarity coefficient cut-off was set at 80%. Optimization and band position tolerance were both set at 0.6%.Representative isolates of each PFGE type and subtype were studied to determine the Multilocus Sequence Type (MLST) [14] and the Staphylococcal Chromosome Cassette *mec* (SCC*mec*) types [15]. MLSTs and SCC*mec* types were further inferred for all the strains. The *agr* polymorphism and the presence of genes encoding class S (*lukS-PV*) and class F (*lukF-PV*) proteins for Panton–Valentine Leucocidine (PVL) were studied by PCR in all the isolates, following the methodology described elsewhere [16,17].

Statistical analysis

Continuous variables were compared using the Student's *t*-test or the Mann–Whitney *U*-test as appropriate. Qualitative and stratified continuous variables were compared using Fisher's exact test or Pearson's chi-squared test. Relative risks were calculated with 95% confidence intervals in a univariate analysis. Time until death was the dependent variable in a Cox regression analysis, and was censored 30 days after the end of therapy or the end of follow-up. Administration of appropriate antibiotic therapy was the dependent variable in a logistic regression model. All the variables with theoretical clinical significance and those that achieved a p value <0.10 in the univariate analysis were included in the multivariate analysis, and adjusted odds ratios (ORs) were calculated with 95% CI. Analyses were performed using SPSS v15 (Microsoft, USA).

Ethical considerations

The study was approved by the Spanish Network for Research in Infectious Diseases (REIPI) as well as the Institutional Review Board of each participating centre. Because no direct patient contact was planned, the requirement for informed consent was waived. The data were de-identified in each centre and only then transferred for analysis.

Results

Five hundred and ninety episodes of MRSA-BSI were confirmed. Eleven were excluded from the analysis due to lack of information. Therefore, 579 episodes were finally included, which are summarized in Table 1.

Twenty-five PFGE types were found among 552 available isolates (Fig. 1). A dominant PFGE genotype (pulse-type 2) was found in 368 (67%), all of which belonged to Clonal Complex (CC) 5 (ST125 and ST146). They carried a SCC*mec* element type IV and *agr* type 2. Forty-seven (9%) isolates of clonal type ST228-agr2, a single locus variant of ST5, were considered a separate clone based on its SCC*mec* polymorphism type I. ST22-SCCmecIV-*agr*1 and ST8-SCC*mec*IV-*agr*1 represented 9% (48/552) and 8% (44/552), respectively, of all studied strains. PVL was found to be positive in 15 isolates; 11 of them belonged to ST8, three to ST125 and one to ST714. Among PVL-positive isolates of ST8, six strains out of 11 belonged to the USA300 clone. Vancomycin MIC (microdilution and *E*-test) distribution according to the molecular characterization is summarized in Table 2. Vancomycin MIC $\geq$ 1.5 μg/mL measured by microdilution and *E*-test was observed in 3.3% and 42.9% of the isolates, respectively. Linezolid MIC_{50} and MIC_{90} were 2 and 2 μg/mL and daptomycin MIC_{50} and MIC_{90} were $<$0.5 and 1 μg/mL.

Initial antibiotic therapy was appropriate in 66% of episodes. Factors related to inappropriate initial antibiotic therapy were certain sources of infection (unknown source (OR 4.22), lower respiratory tract (OR 3.55) and skin and soft tissues (OR 2.58)) and Pitt score $\leq$3 (OR 1.68; Table 3). The median delay in starting appropriate antibiotic therapy was 1.8 days (IQR 0–3). Antibiotics were administered for a median of 18 days (IQR 15–27). The source was eradicated or drained in the first 2 days in 35% of cases.

Overall mortality was observed in 179 (32%) episodes. The Cox-regression analysis identified age $>$70 years (HR 1.88), ultimately fatal or rapidly fatal disease (HR 2.16), Pitt $>$3 (HR 3.45), high-risk source (HR 1.85) and inappropriate initial treatment (HR 1.39) as independent predictive factors for mortality. CC5 (HR 0.52) and CC22 (HR 0.45) were identified as protective factors compared with CC8 (Table 4). Vancomycin MIC $\geq$ 1.5 μg/mL, measured either by the microdilution method (HR 1.71) or by *E*-test (HR 0.78), did not show a significant impact on mortality. Neither was vancomycin MIC $\geq$ 2 μg/mL identified as an independent predictor for mortality, regardless of the method used to assess it

TABLE 1. Summary of all episodes of methicillin-resistant *Staphylococcus aureus* bloodstream infection characteristics

		N episodes (%)
Clinical characteristics		***N* = 579**
Age	Mean (SD)	69.1 (14.3)
Gender	Woman	194 (34)
Charlson	Median (ICR)	3 (2–6)
McCabe	Non-fatal	279 (49)
	Ultimately fatal	212 (37)
	Rapidly fatal	82 (14)
Source	Vascular catheter	218 (38)
	Unknown source	94 (16)
	Skin and soft tissues	81 (14)
	Low respiratory tract	70 (12)
	Surgical site infections	36 (6)
	Urinary tract	28 (5)
	Endocarditis	17 (3)
	Osteoarticular	12 (2)
	Suppurative thrombophlebitis	11 (2)
Distant secondary focus		105 (17)
Persistence		93 (23)
Foreign body presence		270 (47)
Acquisition	Nosocomial	338 (59)
	ICU	72 (13)
	Healthcare related	238 (38)
	Previous admission	78 (14)
	Ambulatory assistance	57 (10)
	Haemodialysis	42 (7)
	Long-term care facilities	41 (7)
	Community	20 (4)
Pitt score	Median (ICR)	1 (0–3)
Microbiological studies		***N* = 552**
PFGE type	2	368 (67)
	4	48 (9)
	5	32 (6)
	12	33 (6)
	Other	71 (13)
agr type	II	419 (76)
	I	122 (22)
	III	11 (2)
MLST- SCCmec	CC5*—IV	371 (67)
	ST22—IV	48 (9)
	ST228—I	47 (9)
	ST8—IV	44 (8)
	Other	42 (8)
PVL		15 (3)
Microdilution vancomycin median MIC (ICR) (μg/mL)		0.73 (0.38–3)
E-test vancomycin median MIC (ICR) (μg/mL)		1.21 (0.38–4)
Treatment		***N* = 579**
Source drainage or catheter withdrawal within first 48 hours		205 (35)
Appropriate initial antibiotic	371 (66)	
	Vancomycin	205 (55)
	Linezolid	67 (18)
	Daptomycin	44 (12)
	Teicoplanin	28 (8)
	Cotrimoxazole	10 (3)
	Clindamycin	9 (2)
	Other *	8 (2)
Definitive antibiotic		526 (91)
	Vancomycin	230 (44)
	Linezolid	104 (20)
	Daptomycin	124 (24)
	Teicoplanin	31 (6)
	Cothrimoxazole	14 (3)
	Clindamycin	4 (1)
	Other *	10 (2)

ICU, intensive care unit; PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; SCCmec, staphylococcal cassette chromosome; PVL, Panton–Valentine Leukocidin; MIC, minimum inhibitory concentration; ICR, interquartile range; *CC5 includes ST125 and ST146.
*Other antibiotics include: tigecyclin, quinolones and fosfomycin with imipenem.

(microdilution, HR 0.83 (95% CI 0.11–6.05); *E*-test, HR 0.94 (95% CI 0.61–1.46)).

In the adjusted Cox-regression model comparing the initial and definitive antibiotic therapies administered, no significant differences in mortality were found (Table 5a,b).

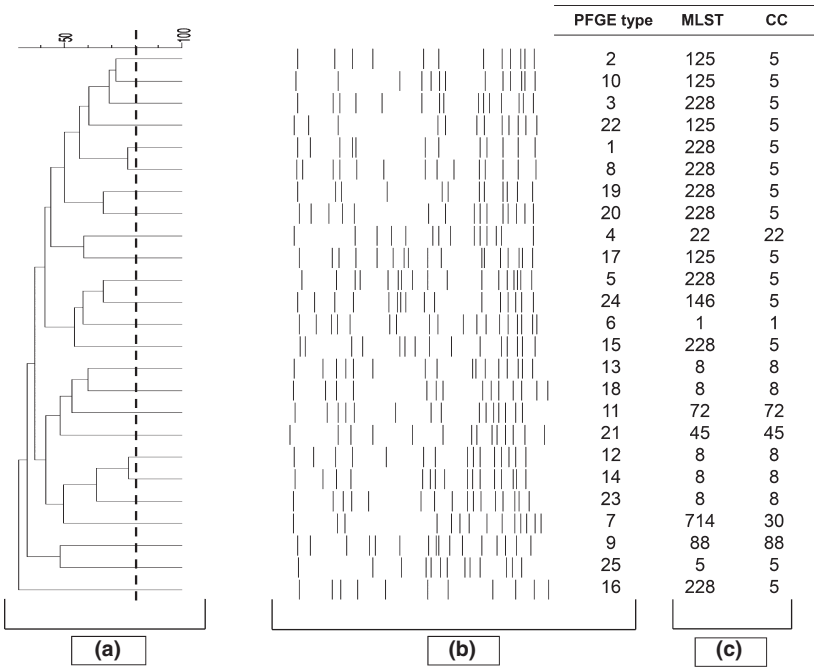
Discussion

This multicentre cohort study reinforces the concept that recent trends towards aging, non-nosocomial acquisition, extended use of new antibiotics and advances in medical support [1,2] have not significantly modified the high mortality rates associated with MRSA-BSI, which remain close to 30% [18]. We analysed the current prognostic factors for mortality, taking into account potential clinical, microbiological and therapeutic predictors. In our opinion, a number of our results merit further discussion.

From a clinical perspective, age, co-morbidities, high-risk sources and severity of sepsis were identified as independent host predictors for mortality. All of these had been identified in previous studies of MRSA-BSI [3,8,19]. A recent review [18] showed age to be the strongest and most consistent predictor for mortality in *S. aureus* bacteraemia. In contrast to other studies [8], healthcare-related acquisition was not identified as a risk factor for worse outcome, probably because of the low mortality rate among episodes acquired during haemodialysis (14%), which represented 19% of the non-nosocomial cohort.

There is little information in the literature regarding the influence of genetic background on the outcome of MRSA infections. It remains unknown whether some MRSA clones have a greater ability to cause invasive disease: some studies have found significant differences between them, but others have not [20,21]. A recent study observed a greater number of haematogenous complications in episodes caused by strains belonging to CC5 and CC30 [22]. Interestingly, in our study CC5 (ST125, ST146 and ST228) and ST22 (EMRSA15) were identified as protective factors compared with CC8. Regarding the PVL gene, in agreement with other authors [23], we found no relationship with worse outcomes. This gene was only harboured by some of the isolates in our study (3%), highlighting the current low prevalence of typical MRSA community clones in Spain [24].

We were particularly interested in assessing the clinical impact of vancomycin susceptibility on mortality. We did not find an independent association with vancomycin MIC $\geq$ 1.5 μg/mL, measured either by microdilution or by *E*-test. These results contrast with those of other studies



PFGE type	MLST	CC
2	125	5
10	125	5
3	228	5
22	125	5
1	228	5
8	228	5
19	228	5
20	228	5
4	22	22
17	125	5
5	228	5
24	146	5
6	1	1
15	228	5
13	8	8
18	8	8
11	72	72
21	45	45
12	8	8
14	8	8
23	8	8
7	714	30
9	88	88
25	5	5
16	228	5

FIG. 1. Molecular characterization of MRSA isolates. Shown from left to right are (a) a dendrogram comparing pulsed-field gel electrophoresis (PFGE) of *Sma*I macrorestriction fragments, (b) PFGE patterns and types (expressed by numbers), followed by (c) multilocus sequence typing (MLST) and clonal complex (CC). The cut-off value to define PFGE types was set at 80%. Optimization and band position tolerance were both set at 0.6%.

TABLE 2. Distribution of vancomycin MIC (μg/mL) tested by microdilution and *E*-test methods among the three major clonal complexes in the cohort of methicillin-resistant *Staphylococcus aureus* bloodstream infections

	Vancomycin MIC (μg/mL)							
	≤0.5	0.75	1	1.5	2	3	4	Total
CC 5 (SCC*mec* type IV, *agr* 2)								
Microdilution	38 (10.2)	254 (68.5)	70 (18.9)	7 (1.9)	1 (0.3)	1 (0.3)	0 (0)	371
E-test	13 (3.5)	20 (5.4)	159 (42.9)	134 (36.1)	42 (11.3)	2 (0.5)	1 (0.3)	
ST228 * (SCC*mec* type I, *agr* 2)								
Microdilution	4 (8.6)	29 (61.7)	10 (21.3)	3 (6.4)	1 (2.1)	0 (0)	0 (0)	47
E-test	1 (2.1)	3 (6.4)	9 (19.1)	21 (44.7)	11 (23.4)	2 (4.3)	0 (0)	
ST 8 (SCC*mec* type IV, *agr* 1)								
Microdilution	16 (36.4)	25 (56.8)	2 (4.5)	1 (2.3)	0 (0)	0 (0)	0 (0)	44
E-test	6 (13.6)	13 (29.5)	20 (45.5)	5 (11.4)	0 (0)	0 (0)	0 (0)	
ST 22 (†) (SCC*mec* type IV, *agr* 1)								
Microdilution	37 (77.1)	8 (16.7)	1 (2.1)	2 (4.2)	0 (0)	0 (0)	0 (0)	48
E-test	17 (35.4)	17 (35.4)	9 (18.8)	3 (6.3)	2 (4.2)	0 (0)	0 (0)	
Other clonal complexes								
Microdilution	10 (23.8)	27 (64.3)	3 (7.1)	2 (4.8)	0 (0)	0 (0)	0 (0)	42
E-test	9 (21.4)	3 (7.1)	16 (38.1)	12 (28.6)	2 (4.8)	0 (0)	0 (0)	
Total								
Microdilution	105 (19.0)	343 (62.1)	86 (15.6)	15 (2.7)	2 (0.4)	1 (0.2)	0 (0)	552
E-test	46 (8.3)	56 (10.1)	213 (38.6)	175 (31.7)	57 (10.3)	4 (0.7)	1 (0.2)	

*ST228, single locus variant of ST5.
†ST22, EMRSA15.

that reported an association between higher vancomycin MIC measured by *E*-test and mortality [3] or treatment failure [25] among patients treated with vancomycin. Striking results in two recent studies suggested that decreased vancomycin susceptibility might be a marker of certain unidentified host or strain factors, related to worse outcomes: Aguado *et al.* [26] observed that *E*-test vancomycin MIC $\geq$ 1.5 was the only independent predictive factor for complicated MSSA bacteraemia treated with betalactams, while Holmes *et al.* [27] found increased

TABLE 3. Predictive factors for inappropriate initial therapy for methicillin-resistant *Staphylococcus aureus* bloodstream infection

		Appropriate initial antibiotic *n* (%)	Inappropriate initial antibiotic *n* (%)	Univariate		Multivariate
				OR	p-Value	OR (95% CI)
Age	>70	193 (52.3)	117 (58.2)	1.27	0.18	1.11 (0.76–1.63)
Gender	Female	126 (34.0)	65 (32.3)	0.93	0.65	1.01 (0.68–1.51)
Charlson score	>5	96 (26.1)	65 (32.3)	1.35	0.11	
McCabe	Non-fatal disease	183 (49.7)	94 (47.5)			
	Ultimately fatal	136 (37.0)	71 (35.9)	1.02	0.93	
	Rapidly fatal	49 (13.3)	33 (16.7)	1.31	0.29	
Pitt	≤ 3	287 (77.8)	166 (83.8)	0.68	0.09	1.68 (1.02–2.79)
Foreign body presence		187 (50.4)	80 (39.8)	0.65	0.015	1.27 (0.79–2.03)
Source						
	Skin and soft tissues	47 (12.7)	34 (16.9)	2.37	0.02	2.58 (1.35–4.90)
	Surgical site infection	24 (6.5)	12 (6.0)	1.64	0.2	
	Urinary tract	20 (5.4)	8 (4.0)	1.3	0.55	
	Lower respiratory tract	35 (9.4)	33 (16.4)	3.09	<0.001	3.55 (1.76–7.15)
	Unknown	41 (11.1)	49 (24.4)	3.91	<0.001	4.22 (2.25–7.93)
Distant secondary focus		76 (20.7)	25 (12.5)	0.65	0.015	0.64 (0.37–1.10)
Acquisition	Nosocomial	222 (60.0)	111 (55.8)			
	Non-nosocomial*	148 (40.0)	88 (44.2)	1.19	0.33	

*Non-nosocomial acquisition includes healthcare-related and community acquisitions.

TABLE 4. Predictive factors for mortality due to methicillin-resistant *Staphylococcus aureus* bloodstream infection. Univariate and multivariate analyses

		30-day deaths *n* (%)	30-day survivors *n* (%)	Univariate		Multivariate analysis with microdilution vancomycin MIC $\geq$ 1.5	Multivariate analysis with *E*-test vancomycin MIC $\geq$ 1.5
				HR	p-Value	HR (95% CI)	HR (95% CI)
Clinical characteristics							
Age	>70	121(68.0)	183(48.4)	1.80	<0.001	1.88 (1.39–2.54)	1.89 (1.40–2.54)
Gender	Woman	64 (35.8)	121(32.0)	1.12	0.43	1.31 (0.98–1.77)	1.29 (0.97–1.74)
Charlson	>5	63 (35.2)	94 (25.0)	1.52	0.003	1.12 (0.82–1.53)	1.15 (0.85–1.57)
McCabe	Non-fatal	58 (21.6)	211(58.4)				
	Ultimately or rapidly fatal	119(67.2)	163(43.6)	2.39	<0.001	2.16 (1.56–2.98)	2.04 (1.49–2.81)
Pitt score	>3	73 (41.7)	40 (10.6)	3.48	<0.001	3.45 (2.57–4.76)	3.66 (2.69–4.98)
Acquisition	Non-nosocomial *	74 (41.6)	157(41.8)	0.91	0.47		
High-risk Source		86 (48.0)	86 (22.8)	2.13	<0.001	1.85 (1.33–2.56)	1.77 (1.28–2.46)
	Endocarditis	8 (4.5)	9 (2.4)	1.60	0.042		
	Low respiratory tract	32 (17.9)	35 (9.3)	1.77	0.002		
	Unknown	46 (25.7)	42 (11.1)	1.89	<0.001		
Foreign body presence		73 (40.8)	189(50.0)	0.76	0.045	0.82 (0.59–1.14)	0.84 (0.61–1.10)
Distant secondary focus		29 (16.4)	71 (18.9)	0.79	0.167	0.92 (0.63–1.34)	0.93 (0.64–1.36)
Microbiological studies							
agr type	I	42 (24.1)	75 (21.1)				
	II	129(74.1)	274(77.0)	0.86	0.34		
	III	3 (1.7)	7 (2.0)	0.70	0.56		
PFGE type	Other	24 (13.8)	40 (11.2)				
	4	12 (6.9)	36 (10.1)	0.65	0.17		
	5	10 (5.7)	20 (5.6)	1.01	0.97		
	2	112(64.4)	244(68.5)	0.85	0.44		
	12	16 (9.4)	16 (4.5)	1.49	0.19		
Clonal complex	CC8	20 (11.7)	23 (6.6)	1.25	0.52		
	CC5 †	129(75.0)	271(77.7)	0.64	0.049	0.52 (0.33–0.83)	0.58 (0.36–0.94)
	CC22	12 (7.0)	37 (10.6)	0.50	0.032	0.45 (0.22–0.89)	0.46 (0.23–0.91)
	Other	12 (7.0)	21 (6.0)	0.75	0.40		
PVL		5 (2.9)	9 (2.5)	1.00	0.99		
Microdilution vancomycin MIC $\geq$ 1.5		7 (4.0)	11 (3.1)	1.56	0.13	1.71 (0.92–3.19)	
Microdilution vancomycin MIC $\geq$ 2		1 (0.6)	2 (0.6)	0.78	0.76		
E-test vancomycin MIC $\geq$ 1.5		69 (39.7)	160(44.9)	0.78	0.08		0.78 (0.58–1.05)
E-test vancomycin MIC $\geq$ 2		20 (11.5)	39 (11.0)	0.85	1.04		
Initial treatment (<48 hours)							
Source drainage or catheter withdrawal		49 (27.4)	153(40.5)	0.64	0.002	0.93 (0.63–1.36)	0.89 (0.61–1.29)
Inppropriate initial antibiotic		72 (41.1)	125(33.2)	1.39	0.014	1.39 (1.04–1.86)	1.37 (1.02–1.83)

PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence type; PVL, Panton–Valentine Leucocidine; MIC, minimum inhibitory concentration (μg/mL).
*Non-nosocomial acquisition includes healthcare-related and community acquisitions.
†CC5 includes ST125, ST146 and ST228.

mortality among episodes of *S. aureus* BSI with MIC $\geq$ 1.5, regardless of the specific antibiotic therapy administered. Like us, however, other authors did not find a significant impact of vancomycin susceptibility on mortality [8,9]. Interestingly, Rojas *et al.* [9] did not observe any association using either susceptibility method.

TABLE 5. **Adjusted multivariate analysis for the appropriate (a) initial (n = 371) and (b) definitive (n = 507) antibiotic therapy administered to patients with methicillin-resistant *Staphylococcus aureus* bloodstream infection**

	30-day deaths *n* (%)	30-day survivors *n* (%)	Unadjusted HR		Adjusted HR * HR (95% CI)
			HR	p	
(a)					
Vancomycin	47 (45.6)	151 (60.2)			
Daptomycin	13 (12.6)	30 (12.0)	1.39	0.37	1.42 (0.83–2.44)
Linezolid	27 (26.2)	35 (13.9)	2.48	0.003	1.25 (0.78–2.1)
Other†	16 (15.5)	35 (13.9)	1.47	0.27	1.46 (0.82–2.61)
(b)					
Vancomycin	58 (42.1)	178 (47.6)			
Daptomycin	31 (23.3)	91 (24.3)	1.08	0.76	0.96 (0.65–1.39)
Linezolid	30 (22.6)	69 (18.4)	1.38	0.23	0.92 (0.06–1.41)
Other†	16 (12.0)	36 (9.6)	1.41	0.31	1.38 (0.81–2.35)

*Model adjusted by age, gender, McCabe, source, Pitt, clonal complex.
†Other appropriate antibiotics according to the susceptibility tests.

In our opinion, these contradictory results between studies might have two complementary explantions: first, according to our results and the findings of Holmes *et al.* [27], certain clones might have an independent association with mortality. Therefore, because vancomycin MIC differs from one clone to another, it is reasonable to hypothesize that the distinct clonal distribution in countries can influence the impact of vancomycin susceptibility observed in different studies. Secondly, the low correlation between *E*-test and microdilution, which is illustred in Table 2 [28], might also lead to these different observed results. In our opinion, as Rojas *et al.* [9] stressed, there is lack of evidence in favour of using the *E*-test instead of the recommended microdilution method to assess MRSA vancomycin MIC. As far as we know, only one previous study found an association between vancomycin MIC $\geq$ 2 μg/mL and mortality, using the microdilution method [19]. A recent meta-analysis of MRSA BSI studies that assessed vancomycin MICs by *E*-test concluded that while MIC $\geq$ 1.5 μg/mL was not associated with higher mortality, MIC $\geq$ 2 was [7]. In contrast, our study and others [29] did not find an independent association with *E*-test vancomycin MICs $\geq$2. In summary, we believe that more studies will be needed to resolve this controversy.

As regards therapeutic management, the initial therapy was inappropriate in 34% of the episodes. It was also identified as an independent predictive factor for mortality, as in previous studies [3,30]. This is an interesting observation because modifying this factor might reduce the number of related deaths. In our study, patients with lower Pitt scores and specific sources had a significantly higher risk of receiving initial inappropriate antibiotic therapy. Interestingly, we found no statistical differences in mortality in relation to the type of appropriate antibiotics used as initial or definitive therapy. However, we stress that this last analysis is limited because the choice of antibiotic was not controlled and there may have been some confounding factors in the variables regarding the therapy. Also, vancomycin serum levels were not assessed in this study. Therefore, conclusions regarding the impact of antibiotic therapy should be reached with caution.

In conclusion, we identified several prognostic factors for mortality in a large cohort of patients and provide support for the concept that host and bacterial characteristics, as well as initial therapy, have implications for MRSA-BSI outcomes. In contrast, higher vancomycin MIC had no impact on mortality, regardless of the method used to assess it. Special attention should be paid to patients with one or some of these predictors, in order to ensure the administration of correct initial therapy.

Acknowledgements

This work supported by Ministerio de Innovación, Instituto de Salud Carlos III (FIS 08/0335), and co-financed by European development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008). O.G had agrant Rio Hortega (CM08/228) from the Instituto de Salud Carlos III. A part of the results of this article was presented at the 51st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, 16–20 September, 2011. Presentation number: K-950.

Transparency Declaration

B.A received funding for research from Pfizer, Novartis, Gilead y MSD and funds for advisory board membership Pfizer, Gilead, Novartis, Janssen, Astellas and MSD. N.B. received funding for speaking, consultancy, advisory board membership, travel from MSD, Pfizer, Gilead, Novartis, AstraZeneca. J.R.B. received funding for research from Novartis, has served as speaker for Astellas, Merck, AstraZeneca, and Pfizer, and has

been consultant for Roche, Novartis, and Janssen. The rest of the authors have no conflicts of interest to declare.

Appendix

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References

1. Klevens RM, Morrison MA, Nadle J *et al.* Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* 2007; 298: 1763–1771.
2. Gasch O, Ayats J, Angeles DM *et al.* Epidemiology of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Bloodstream Infection: Secular Trends Over 19 Years at a University Hospital. *Medicine (Baltimore)* 2011; 90: 319–327.
3. Soriano A, Marco F, Martinez JA *et al.* Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Clin Infect Dis* 2008; 46: 193–200.
4. Small PM, Chambers HF. Vancomycin for *Staphylococcus aureus* endocarditis in intravenous drug users. *Antimicrob Agents Chemother* 1990; 34: 1227–1231.
5. Sader HS, Fey PD, Limaye AP *et al.* Evaluation of vancomycin and daptomycin potency trends (MIC creep) against methicillin-resistant *Staphylococcus aureus* isolates collected in nine U.S. medical centers from 2002 to 2006. *Antimicrob Agents Chemother* 2009; 53: 4127–4132.
6. Stevens DL. The role of vancomycin in the treatment paradigm. *Clin Infect Dis* 2006; 42 (Suppl 1): S51–S57.
7. van Hal SJ, Lodise TP, Paterson DL. The clinical significance of vancomycin minimum inhibitory concentration in *Staphylococcus aureus* infections: a systematic review and meta-analysis. *Clin Infect Dis* 2012; 54: 755–771.
8. Pastagia M, Kleinman LC. Lacerda de la Cruz EG, Jenkins SG. Predicting risk for death from MRSA bacteremia(1). *Emerg Infect Dis* 2012; 18: 1072–1080.
9. Rojas L, Bunsow E, Munoz P, Cercenado E, Rodriguez-Creixems M, Bouza E. Vancomycin MICs do not predict the outcome of methicillin-resistant *Staphylococcus aureus* bloodstream infections in correctly treated patients. *J Antimicrob Chemother* 2012; 67: 1760–1768.
10. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections, 1988. *Am J Infect Control* 1988; 16: 128–140.
11. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: twenty-first informational supplement M100-S21*. Wayne, PA, USA: CLSI, 2011.
12. Chung M, de Lencastre H, Matthews P *et al.* Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist* 2000; 6: 189–198.
13. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
14. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; 38: 1008–1015.
15. Milheirico C, Oliveira DC, de Lencastre H *et al.* Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2007; 51: 3374–3377.
16. Shopsin B, Mathema B, Alcabes P *et al.* Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J Clin Microbiol* 2003; 41: 456–459.
17. Lina G, Piemont Y, Godail-Gamot F *et al.* Involvement of Panton–Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 1999; 29: 1128–1132.
18. van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB. Predictors of Mortality in *Staphylococcus aureus* Bacteremia. *Clin Microbiol Rev* 2012; 25: 362–386.
19. Wang JL, Wang JT, Sheng WH, Chen YC, Chang SC. Nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia in Taiwan: mortality analyses and the impact of vancomycin, MIC = 2 mg/L, by the broth microdilution method. *BMC Infect Dis* 2010; 10: 159.
20. Gill SR, McIntyre LM, Nelson CL *et al.* Potential associations between severity of infection and the presence of virulence-associated genes in clinical strains of *Staphylococcus aureus*. *PLoS ONE* 2011; 6: e18673.
21. Jarraud S, Mougel C, Thioulouse J *et al.* Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun* 2002; 70: 631–641.
22. Fowler VG Jr, Nelson CL, McIntyre LM *et al.* Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *J Infect Dis* 2007; 196: 738–747.
23. Lalani T, Federspiel JJ, Boucher HW *et al.* Associations between the genotypes of *Staphylococcus aureus* bloodstream isolates and clinical characteristics and outcomes of bacteremic patients. *J Clin Microbiol* 2008; 46: 2890–2896.

24. Rodriguez-Bano J, Bischofberger C, Alvarez-Lerma F *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: 285–298.
25. Moise PA, Sakoulas G, Forrest A, Schentag JJ. Vancomycin in vitro bactericidal activity and its relationship to efficacy in clearance of methicillin-resistant *Staphylococcus aureus* bacteremia. *Antimicrob Agents Chemother* 2007; 51: 2582–2586.
26. Aguado JM, San-Juan R, Lalueza A *et al.* High vancomycin MIC and complicated methicillin-susceptible *Staphylococcus aureus* bacteremia. *Emerg Infect Dis* 2011; 17: 1099–1102.
27. Holmes NE, Turnidge JD, Munckhof WJ *et al.* Antibiotic choice may not explain poorer outcomes in patients with *Staphylococcus aureus* bacteremia and high vancomycin minimum inhibitory concentrations. *J Infect Dis* 2011; 204: 340–347.
28. Hsu DI, Hidayat LK, Quist R *et al.* Comparison of method-specific vancomycin minimum inhibitory concentration values and their predictability for treatment outcome of meticillin-resistant *Staphylococcus aureus* (MRSA) infections. *Int J Antimicrob Agents* 2008; 32: 378–385.
29. van Hal SJ, Jones M, Gosbell IB, Paterson DL. Vancomycin heteroresistance is associated with reduced mortality in ST239 methicillin-resistant *Staphylococcus aureus* blood stream infections. *PLoS ONE* 2011; 6: e21217.
30. Paul M, Kariv G, Goldberg E *et al.* Importance of appropriate empirical antibiotic therapy for methicillin-resistant *Staphylococcus aureus* bacteraemia. *J Antimicrob Chemother* 2010; 65: 2658–2665.

Journal of Antimicrobial Chemotherapy Advance Access published February 12, 2013

J Antimicrob Chemother
doi:10.1093/jac/dkt016

Journal of Antimicrobial Chemotherapy

Predictive factors for early mortality among patients with methicillin-resistant *Staphylococcus aureus* bacteraemia

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Received 29 November 2012; returned 17 December 2012; revised 30 December 2012; accepted 8 January 2013

Objectives: A high proportion of patients with methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia die within a few days of the onset of infection. However, predictive factors for early mortality (EM) have barely been examined. The aim of this study was to determine the predictive factors for EM in patients with MRSA bacteraemia.

Methods: All episodes of MRSA bacteraemia were prospectively followed in 21 Spanish hospitals from June 2008 to December 2009. Epidemiology, clinical data, therapy and outcome were recorded. All MRSA strains were analysed in a central laboratory. Mortality was defined as death from any cause occurring in the 30 days after the onset of MRSA bacteraemia. EM was defined as patients who died within the first 2 days, and late mortality (LM) for patients who died after this period. Multivariate analyses were performed by using logistic regression models.

Results: A total of 579 episodes were recorded. Mortality was observed in 179 patients (31%): it was early in 49 (8.5%) patients and late in 130 (22.5%). Independent risk factors for EM were [OR (95% CI)] initial Pitt score >3 [3.99 (1.72–3.24)], previous rapid fatal disease [3.67 (1.32–10.24)], source of infection lower respiratory tract or unknown [3.76 (1.31–10.83) and 2.83 (1.11–7.21)], non-nosocomial acquisition [2.59 (1.16–5.77)] and inappropriate initial antibiotic therapy [3.59 (1.63–7.89)]. When predictive factors for EM and LM were compared, inappropriate initial antibiotic therapy was the only distinctive predictor of EM, while endocarditis and lower respiratory tract sources both predicted LM.

Conclusions: In our large cohort of patients several factors were related to EM, but the only distinctive predictor of EM was inappropriate initial antibiotic therapy.

Keywords: MRSA, bloodstream infections, empirical antibiotic therapy

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia is currently a cause of concern in Spain, where the incidence is 7/100 000 patient days and the percentage of methicillin resistance is 30.[1] Mortality among patients with MRSA bacteraemia is close to 30%.[2–4] Predictive factors for mortality have been related to host and microorganism characteristics and the interaction between them, and to therapeutic interventions.[5,6] The impact of host factors on mortality has been extensively analysed; age and comorbidities are the factors identified most frequently.[7] Regarding the interaction between host and MRSA, certain sources of bacteraemia have also been associated with mortality.[8] Some studies have also noted the significance of the setting of acquisition.[9,10] The influence of high MRSA vancomycin MIC on mortality has been widely debated in recent years.

While some studies report worse outcomes in episodes caused by strains with higher vancomycin MICs, even in the susceptible range,[8,11] others do not.[12–16] The impact of distinct MRSA clones on mortality has received little attention.[17,18] Lastly, inappropriate empirical therapy has been described as a predictor of mortality,[19,20] but some studies did not find a significant relationship with poor outcome.[21]

Although a large proportion of deaths occur within the first few days after the onset of *S. aureus* bacteraemia,[2,22] predictors of early mortality (EM) have barely been examined.[23] The objective of this study was to determine distinctive predictors of EM in a large cohort of patients with MRSA bacteraemia.

Patients and methods

Study period and patients

The study was conducted from June 2008 to December 2009 in 21 Spanish hospitals. Four centres had <500 beds, nine had 500–1000 beds and eight had >1000 beds. All consecutive episodes of MRSA bacteraemia observed in adult patients in participating centres were prospectively followed using a standardized protocol. Cases that did not meet the inclusion criteria because of a lack of signs and symptoms consistent with sepsis were excluded. Strains were sent to a central laboratory for further studies.

Study design

Patients with MRSA bacteraemia were classified into three groups according to outcome: (i) patients with EM; (ii) patients with late mortality (LM); and (iii) survivors. With the data, we carried out univariate and multivariate analyses to assess the predictive factors for EM. Then, following the methodology described by Harris *et al.*,[24] two separate analyses were performed: the first compared patients with EM and survivors and the second compared patients with LM and survivors. Variables with statistical significance in the first analysis but not in the second were considered distinctive factors for EM; those that were significant only in the second analysis were considered distinctive predictors of LM.

Definitions

MRSA bacteraemia was defined as the presence of at least one positive blood culture for MRSA in a blood sample from a patient with clinical findings consistent with infection.[25] Comorbidity was measured by the Charlson score,[26] which stratifies the associated diseases on an ordinal scale. Patients were classified into three categories on the McCabe scale,[27] according to their vital prognosis before the MRSA bacteraemia: non-fatal if death was expected within a period of >5 years; ultimately fatal if death was expected between 1 and 5 years; and rapidly fatal if it was expected within the following year. Severity of sepsis in the acute condition was assessed by the Pitt score.[28] According to the Friedman criteria,[29] three acquisition categories were considered: (i) nosocomial bacteraemia if the episode was diagnosed at least 48 h after admission to an ICU or a conventional (non-ICU) hospital ward and if there were no signs or symptoms of infection at admission; (ii) healthcare-related bacteraemia if previous contact with the healthcare system was recorded within the previous 3 months; and (iii) community acquisition if the episode did not fit the previous conditions. The source of the bacteraemia was defined according to the CDC criteria.[30] Bacteraemia from an unknown source (primary) was defined when the origin was uncertain after careful examination of the clinical and microbiological data. Distant extension was diagnosed with the presence of at least one distant infection secondary to blood spread seeding. The initial antibiotic was defined as the antibiotic administered in the first 48 h after the onset of bacteraemia, independently of the microbiological information. Initial antibiotic treatment was considered appropriate if the strain was susceptible to at least one of the antibiotics administered according to the current CLSI breakpoints,[31] with the exception of aminoglycosides, which were considered inappropriate regardless of the susceptibility test results. Source was considered eradicated if the catheter or foreign body was removed, if surgery on the source was carried out or if the bacteraemia source was drained. For outcomes, mortality was defined as in-hospital death from any cause occurring in the 30 days after the onset of MRSA bacteraemia. Mortality was defined as EM for patients who died within the first 2 days and LM for patients who died later.

Susceptibility testing and molecular epidemiology of MRSA isolates

Each hospital identified the strain and performed preliminary susceptibility tests. Isolates were sent to a central reference laboratory. All *S. aureus* were identified by latex agglutination (Pastorex Staph-plus, Bio-Rad Laboratories, Madrid, Spain) and DNase production (DNasE-test Agar, bioMérieux, Marcy l'Étoile, France). Antimicrobial susceptibility of all MRSA isolates was tested by the disc diffusion method according to the CLSI guidelines.[31] The antimicrobial agents tested were penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, gentamicin, tobramycin, ciprofloxacin, rifampicin, trimethoprim/sulfamethoxazole, tetracycline, fosfomycin, vancomycin, teicoplanin, chloramphenicol, daptomycin and linezolid. MICs were determined by the microdilution method in accordance with CLSI criteria by using commercial panels (ESTEN 2009, Sensititre™, Izasa, Barcelona, Spain) read visually. Vancomycin MICs were also determined by Etest (bioMérieux) on Mueller–Hinton agar, using a turbidity equivalent to that of a 0.5 McFarland standard.

PFGE was performed after SmaI restriction of chromosomal DNA.[32] Restriction patterns were interpreted in accordance with criteria published elsewhere.[33] Representative isolates of each PFGE type and subtype were studied to determine the multilocus sequence type (MLST)[34] and the staphylococcal chromosome cassette *mec* (SCC*mec*) type.[35] MLSTs and SCC*mec* types were further inferred for all the strains. The *agr* polymorphism and the presence of genes encoding class S (*lukS-PV*) and class F (*lukF-PV*) proteins for Panton–Valentine leucocidin (PVL) were studied by PCR in all the isolates, following the methodology described elsewhere.[36,37]

Statistical analysis

Continuous variables were compared using Student's *t*-test or the Mann–Whitney *U*-test as appropriate. Qualitative and stratified continuous variables were compared using Fisher's exact test or Pearson's χ^2 test. Relative risks were calculated with 95% CIs in a univariate analysis. The multivariate analyses were performed by using logistic regression models. All the variables with theoretical clinical significance and those that achieved a *P* value <0.10 in the univariate analysis were included in the multivariate analysis and adjusted ORs were calculated with 95% CIs. Analyses were performed using SPSS v15 (Microsoft, USA).

Ethics considerations

The study was approved by the Spanish Network for Research in Infectious Diseases (REIPI) and by the Institutional Review Board at each participating centre. Because no direct patient contact was planned, the requirement for informed consent was waived. The data were de-identified in each centre and only then transferred for analysis.

Results

Overall, 590 episodes of MRSA bacteraemia were recorded. Eleven of them were excluded due to the lack of complete

information. Therefore, 579 episodes were finally included in the analysis. One hundred and seventy-nine patients (31%) died: 49 (8.5%) were defined as EM and 130 (22.5%) as LM. There were 400 (69%) survivors.

Risk factors for EM

The cohort of patients with EM ($n=49$) was compared with the rest ($n=530$). Clinical and microbiological characteristics and therapeutic interventions were included in the univariate and multivariate analyses. Independent risk factors for EM according to logistic regression analysis were rapidly fatal disease (OR 3.67; $P=0.012$), respiratory and unknown sources (OR 3.76; $P=0.014$ and 2.83; $P=0.029$ respectively), non-nosocomial acquisition (OR 2.59; $P=0.021$), Pitt score of ≥3 (OR 3.99; $P=0.001$) and inappropriate initial antibiotic therapy within the first 2 days (OR 3.59; $P=0.001$). No microbiological characteristics, such as *agr* type, clonal complex characterization, PVL or vancomycin MIC, were significantly related to EM (Table 1).

Distinctive predictive factors for EM and LM

Multivariate analysis to identify distinctive predictors for EM and LM are shown in Tables 2 and 3. Survivors and non-survivors were compared. Patients with EM ($n=49$) were more likely to be aged >70 years than survivors (OR 2.77; $P=0.026$), more likely to have

Table 1. Factors independently associated with EM in a logistic regression model of all patients with MRSA bacteraemia

		Early deaths, $n=49$ *n* (%)	Non-early deaths, $n=530$ *n* (%)	Univariate		Multivariate adjusted OR (95% CI)
				OR	*P* value	
Clinical characteristics						
age (years)	>70	33 (67)	283 (54)	1.79	0.07	1.46 (0.66–3.24)
gender	female	15 (31)	179 (34)	0.87	0.65	1.05 (0.48–2.33)
Charlson score	>5	15 (31)	148 (28)	1.13	0.71	
McCabe scale	non-fatal	14 (30)	265 (50)			
	ultimately fatal	20 (43)	192 (37)	1.97	0.06	
	rapidly fatal	13 (28)	69 (13)	3.57	0.02	3.67 (1.32–10.24)
source	skin and soft tissues	4 (8)	77 (15)	1.57	0.48	
	surgical site infection	2 (4)	34 (6)	1.77	0.49	
	catheter	7 (14)	211 (40)			
	endocarditis	1 (2)	16 (3)	1.89	0.57	
	lower respiratory tract	13 (27)	57 (11)	6.88	<0.01	3.76 (1.31–10.83)
	unknown source	17 (35)	77 (15)	6.67	<0.01	2.83 (1.11–7.21)
distant secondary focus		3 (6)	99 (19)	0.28	0.04	
foreign body presence		16 (33)	253 (48)	0.53	0.05	
acquisition	nosocomial	20 (42)	318 (60)			
	non-nosocomial[a]	28 (58)	210 (40)	2.12	0.01	2.59 (1.16–5.77)
Pitt score	>3	24 (49)	92 (18)	4.52	<0.01	3.99 (1.72–9.24)
Microbiological studies						
agr type	I	15 (31)	108 (21)			
	II	32 (67)	386 (77)	0.59	0.12	
	III	1 (2)	10 (2)	0.72	0.76	
PFGE type	12	9 (19)	24 (5)			
	4	1 (2)	47 (9)	0.57	<0.01	
	5	1 (2)	31 (6)	0.09	0.02	
	2	30 (63)	341 (68)	0.24	0.01	
clonal complex	5[b]	32 (67)	385 (78)			
	8	9 (19)	35 (7)	3.09	<0.01	
	22	1 (2)	46 (9)	0.26	0.19	
	other	6 (13)	28 (6)	2.58	0.06	
PVL		1 (2)	14 (3)	0.75	0.78	
vancomycin MIC	≥1.5 mg/L	4 (8)	14 (3)	3.18	0.05	
Initial treatment (<48 h)						
source drainage		6 (12)	199 (38)	0.23	<0.01	
inappropriate initial antibiotic		31 (67)	170 (32)	4.33	<0.01	3.59 (1.63–7.89)

[a]Non-nosocomial acquisition includes healthcare-related and community acquisitions.
[b]Clonal complex 5 (CC5) includes ST125, ST146 and ST228.

Table 2. Associated factors for EM (compared with 30 day survivors); logistic regression model

		Univariate *P* value	Multivariate adjusted OR (95% CI)
Clinical characteristics			
age (years)	>70	0.01	2.77 (1.11–6.89)
gender	female	0.84	
Charlson score	>5	0.39	
McCabe scale	non-fatal		
	ultimately fatal	0.03	
	rapidly fatal	<0.01	10.38 (3.13–34.4)
Pitt score	>3	<0.01	13.36 (4.46–39.9)
acquisition	nosocomial		
	non-nosocomial[a]	0.031	
source	vascular catheter		
	skin and soft tissues	0.51	
	surgical site infection	0.46	
	endocarditis	0.40	
	lower respiratory tract	<0.01	
	unknown	<0.01	5.16 (1.67–15.9)
foreign body presence		0.03	
Microbiological studies			
agr type	I		
	II	0.11	
	III	0.76	
PFGE type	12		
	4	0.06	
	5	0.03	
	2	0.01	
clonal complex	5[b]		
	8	<0.01	
	22	0.17	
	other	0.08	
PVL		0.85	
vancomycin MIC	≥1.5 (mg/L)	0.08	
Initial treatment (<48 h)			
source drainage		<0.01	
inappropriate initial antibiotic		<0.01	3.88 (1.55–9.73)

[a]Non-nosocomial acquisition includes healthcare-related and community acquisitions.
[b]CC5 includes ST125, ST146 and ST228.

rapidly fatal disease (OR 10.38; $P<0.001$), unknown source (OR 5.16; $P=0.004$), a Pitt score ≥3 (OR 13.36; $P<0.001$) and inappropriate initial antibiotic therapy within the first 2 days (OR 3.88; $P=0.004$). Patients with LM ($n=130$) were more likely to be aged >70 years (OR 3.32; $P<0.001$), to have rapidly fatal disease (OR 8.55; $P<0.001$), endocarditis and lower respiratory tract and unknown sources (OR 4.12; $P=0.011$, OR 2.46; $P=0.039$ and OR 3.07; $P=0.003$, respectively) and a Pitt score of ≥3 (OR 6.07; $P<0.001$) than survivors.

A comparison of the two models showed that for patients with EM inappropriate initial antibiotic therapy within the first 2 days was an independent distinctive factor (Figure 1), while endocarditis and lower respiratory tract sources were distinctive factors for LM. Old age, rapidly fatal disease, unknown source and Pitt score were associated with mortality in both groups.

Discussion

More than 20 years since the dissemination of MRSA strains in Spanish hospitals, our knowledge of this microorganism has significantly improved. However, mortality of patients with MRSA bacteraemia remains high, close to 30%.[38] In this large multicentre, prospective study we found that a considerable proportion of all-cause deaths among patients with MRSA bacteraemia occurred in the first 2 days after onset. Inappropriate antibiotic therapy was identified as a distinctive predictor of EM, and endocarditis and lower respiratory tract sources were identified as predictors of LM.

Although this issue has not been specifically studied to date, we infer that about 50% of non-survivors may die within the first 4–9 days after the onset of staphylococcal bacteraemia.[3,39,40] To our knowledge, there are no studies that analyse in detail the

Table 3. Associated factors for LM (compared with 30 day survivors); logistic regression model

		Univariate *P* value	Multivariate adjusted OR (95% CI)
Clinical characteristics			
age (years)	>70	<0.01	3.32 (1.92–5.73)
gender	woman	0.24	
Charlson score	>5	0.01	
McCabe scale	non-fatal		
	ultimately fatal	0.02	
	rapidly fatal	<0.01	8.55 (4.08–17.91)
Pitt score	>3	<0.01	6.07 (3.33–11.07)
acquisition	nosocomial		
	non-nosocomial[a]	0.20	
source	vascular catheter		
	skin and soft tissues	0.23	
	surgical site infection	0.59	
	endocarditis	0.06	4.12 (1.48–11.45)
	lower respiratory tract	0.05	2.46 (1.04–5.79)
	unknown	0.003	3.07 (1.46–6.45)
foreign body presence		0.23	
Microbiological studies			
agr type	I		
	II	0.95	
	III	0.78	
PFGE type	12		
	4	0.53	
	5	0.96	
	2	0.58	
clonal complex	5[b]		
	8	0.55	
	22	0.72	
	other	0.64	
PVL		0.70	
vancomycin MIC	≥1.5 (mg/L)	0.69	
Initial treatment (<48 h)			
source drainage		0.14	
inappropriate initial antibiotic		0.76	

[a]Non-nosocomial acquisition includes healthcare-related and community acquisitions.
[b]CC5 includes ST125, ST146 and ST228.

factors associated with EM. However, a recent population study from the UK found a rate of EM of 11% and reported that the highest risk of death (4.5%) was during the first day of bacteraemia.[23] The absence of clinical, microbiological and treatment data in that study limits the possibility of further analysis regarding the risk factors for EM.

Studies that have examined the relationship between inappropriate empirical therapy and mortality in *S. aureus* and MRSA bacteraemia have yielded conflicting results. Although some studies did not find a significant association,[21,41,42] many others did: Soriano *et al.*[10] identified inappropriate therapy as a predictor of related mortality, with an OR of 2.13, as did Rodríguez-Baño *et al.*[20] in a cohort of healthcare-acquired sepsis due to MRSA (OR 3.0). Marchaim *et al.*[43] identified a delay of 2 days in receiving appropriate antibiotic therapy as a predictor of in-hospital mortality (OR 2.35) and Paul *et al.*[19] reported a significant association with 30 day mortality. Lodise and McKinnon[4] also found an association with 30 day mortality (OR 2.1) and infection-related death (OR 2.2). Lastly, a delay in adequate antibiotic therapy of >44.76 h was identified as an independent predictor of related mortality (OR 3.8) in patients with *S. aureus* bacteraemia.[22] In the study by Lodise and McKinnon,[4] MRSA was an independent predictor of delayed therapy. In our opinion, the identification of inappropriate initial antibiotic therapy as the distinctive predictor of EM supports the hypothesis that the use of 30 day mortality rate may be an excessively crude way of assessing the impact of initial antibiotic therapy.

Our results reinforce the importance of ensuring early administration of adequate antibiotic treatment when MRSA bacteraemia is a diagnostic possibility. As in other studies,[19] a great

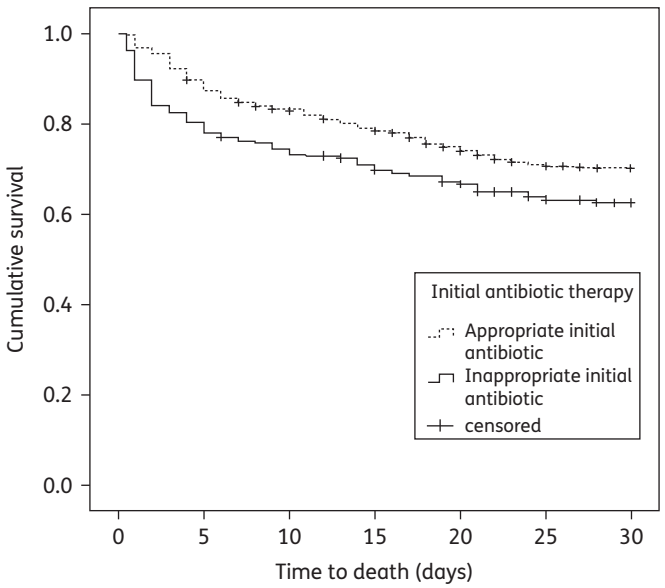

Figure 1. Survival analysis of MRSA bacteraemia episodes according to the administration of appropriate initial antibiotic therapy.

proportion of patients in our series received inappropriate coverage, especially when acquisition was non-nosocomial (data not shown). This is a striking observation since risk factors for MRSA bacteraemia at hospital admission are well established,[44,45] the most significant one being referral from a long-term care facility and prior MRSA colonization. This information should be taken into account to avoid unnecessary delays in the administration of appropriate antibiotic therapy.

Our study has some limitations. First, our definition of appropriate antibiotic therapy is controversial: antibiotics classified as inadequate, such as aminoglycosides, may have some effect on MRSA. Also, glycopeptides were classified as appropriate regardless of serum levels, which were not always assessed in the first 48 h. In contrast, some bacteriostatic antibiotics classified as appropriate would not usually be used as first choice against MRSA bacteraemia, as other authors have previously pointed out.[46,47] Second, differences between centres regarding the clinical management and early suspicion of the infection as well as time until the microbiological identification of susceptibility patterns and the presence of the gene *mecA* may confound the evaluation of antibiotic therapy during the first 2 days.

In summary, our experience suggests that inappropriate antibiotic treatment plays a crucial role in EM among patients with MRSA bacteraemia. Strategies to ensure the adequacy of empirical antibiotic therapy are needed.

Acknowledgements

Writing assistance was provided by the Department of Languages in the Faculty of Medicine of Barcelona University.

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Funding

This study was supported by Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III (FIS 08/0335) and co-financed by the European Development Regional Fund 'A way to achieve Europe' ERDF, Spanish Network for Research in Infectious Diseases (REIPI RD06/0008).

O. G. is the recipient of a Río Hortega grant (CM08/228) from the Instituto de Salud Carlos III.

Transparency declarations

B. A. has received funding for research from Pfizer, Novartis, Gilead and MSD, and funds for advisory board membership from Pfizer, Gilead, Novartis, Janssen, Astellas and MSD. N. B. has received funding for speaking, consultancy, advisory board membership and travel from MSD, Pfizer, Gilead, Novartis and AstraZeneca. J. R.-B. has received funding for research from Novartis, has served as speaker for Astellas, Merck, AstraZeneca and Pfizer, and has been a consultant for Roche, Novartis and Janssen. All other authors: none to declare.

References

1 European Centre for Disease Prevention and Control (ECDC). *Annual Epidemiological Report on Communicable Diseases in Europe*. 2010. Stockholm: ECDC. http://www.ecdc.europa.eu/en/eaad/documents/eaad-2011-summary-antimicrobial-resistance-data.pdf (28 December 2012, date last accessed).

2 Benfield T, Espersen F, Frimodt-Moller N *et al*. Increasing incidence but decreasing in-hospital mortality of adult *Staphylococcus aureus* bacteraemia between 1981 and 2000. *Clin Microbiol Infect* 2007; **13**: 257–63.

3 Kaech C, Elzi L, Sendi P *et al*. Course and outcome of *Staphylococcus aureus* bacteraemia: a retrospective analysis of 308 episodes in a Swiss tertiary-care centre. *Clin Microbiol Infect* 2006; **12**: 345–52.

4 Lodise TP, McKinnon PS. Clinical and economic impact of methicillin resistance in patients with *Staphylococcus aureus* bacteremia. *Diagn Microbiol Infect Dis* 2005; **52**: 113–22.

5 Liao CH, Chen SY, Huang YT *et al*. Outcome of patients with meticillin-resistant *Staphylococcus aureus* bacteraemia at an emergency department of a medical centre in Taiwan. *Int J Antimicrob Agents* 2008; **32**: 326–32.

6 Lin SH, Liao WH, Lai CC *et al*. Risk factors for mortality in patients with persistent methicillin-resistant *Staphylococcus aureus* bacteraemia in a tertiary care hospital in Taiwan. *J Antimicrob Chemother* 2010; **65**: 1792–8.

7 van Hal SJ, Jensen SO, Vaska VL *et al*. Predictors of mortality in *Staphylococcus aureus* bacteremia. *Clin Microbiol Rev* 2012; **25**: 362–86.

8 Soriano A, Marco F, Martinez JA *et al*. Influence of vancomycin minimum inhibitory concentration on the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Clin Infect Dis* 2008; **46**: 193–200.

9 Holmes NE, Turnidge JD, Munckhof WJ *et al*. Antibiotic choice may not explain poorer outcomes in patients with *Staphylococcus aureus* bacteremia and high vancomycin minimum inhibitory concentrations. *J Infect Dis* 2011; **204**: 340–7.

10 Soriano A, Martinez JA, Mensa J *et al*. Pathogenic significance of methicillin resistance for patients with *Staphylococcus aureus* bacteremia. *Clin Infect Dis* 2000; **30**: 368–73.

11 Lodise TP, Graves J, Evans A *et al*. Relationship between vancomycin MIC and failure among patients with methicillin-resistant *Staphylococcus aureus* bacteremia treated with vancomycin. *Antimicrob Agents Chemother* 2008; **52**: 3315–20.

12 Rojas L, Bunsow E, Munoz P *et al*. Vancomycin MICs do not predict the outcome of methicillin-resistant *Staphylococcus aureus* bloodstream infections in correctly treated patients. *J Antimicrob Chemother* 2012; **67**: 1760–8.

13 van Hal SJ, Lodise TP, Paterson DL. The clinical significance of vancomycin minimum inhibitory concentration in *Staphylococcus aureus* infections: a systematic review and meta-analysis. *Clin Infect Dis* 2012; **54**: 755–71.

14 Pastagia M, Kleinman LC, Lacerda de la Cruz EG *et al*. Predicting risk for death from MRSA bacteremia. *Emerg Infect Dis* 2012; **18**: 1072–80.

15 Albur MS, Bowker K, Weir I *et al*. Factors influencing the clinical outcome of methicillin-resistant *Staphylococcus aureus* bacteraemia. *Eur J Clin Microbiol Infect Dis* 2012; **31**: 295–301.

16 Honda H, Doern CD, Michael-Dunne W Jr *et al*. The impact of vancomycin susceptibility on treatment outcomes among patients with methicillin resistant *Staphylococcus aureus* bacteremia. *BMC Infect Dis* 2011; **11**: 335.

17 Fowler VG Jr, Nelson CL, McIntyre LM *et al*. Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *J Infect Dis* 2007; **196**: 738–47.

18 Lalani T, Federspiel JJ, Boucher HW *et al*. Associations between the genotypes of *Staphylococcus aureus* bloodstream isolates and clinical characteristics and outcomes of bacteremic patients. *J Clin Microbiol* 2008; **46**: 2890–6.

19 Paul M, Kariv G, Goldberg E *et al*. Importance of appropriate empirical antibiotic therapy for methicillin-resistant *Staphylococcus aureus* bacteraemia. *J Antimicrob Chemother* 2010; **65**: 2658–65.

20 Rodríguez-Baño J, Millán AB, Domínguez MA *et al*. Impact of inappropriate empirical therapy for sepsis due to health care-associated methicillin-resistant *Staphylococcus aureus*. *J Infect* 2009; **58**: 131–7.

21 Roghmann MC. Predicting methicillin resistance and the effect of inadequate empiric therapy on survival in patients with *Staphylococcus aureus* bacteremia. *Arch Intern Med* 2000; **160**: 1001–4.

22 Lodise TP, McKinnon PS, Swiderski L *et al*. Outcomes analysis of delayed antibiotic treatment for hospital-acquired *Staphylococcus aureus* bacteremia. *Clin Infect Dis* 2003; **36**: 1418–23.

23 Lamagni TL, Potz N, Powell D *et al*. Mortality in patients with meticillin-resistant *Staphylococcus aureus* bacteraemia, England 2004–2005. *J Hosp Infect* 2011; **77**: 16–20.

24 Harris AD, Samore MH, Lipsitch M *et al*. Control-group selection importance in studies of antimicrobial resistance: examples applied to *Pseudomonas aeruginosa*, enterococci, and *Escherichia coli*. *Clin Infect Dis* 2002; **34**: 1558–63.

25 Levy MM, Fink MP, Marshall JC *et al*. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 2003; **31**: 1250–6.

26 Charlson ME, Pompei P, Ales KL *et al*. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373–83.

27 McCabe WR, Jackson GG. Gram-negative bacteremia. Clinical, laboratory and therapeutic observations. *Arch Intern Med* 1962; **110**: 856–64.

28 Chow JW, Yu VL. Combination antibiotic therapy versus monotherapy for gram-negative bacteraemia: a commentary. *Int J Antimicrob Agents* 1999; **11**: 7–12.

29 Friedman ND, Kaye KS, Stout JE *et al*. Health care-associated bloodstream infections in adults: a reason to change the accepted definition of community-acquired infections. *Ann Intern Med* 2002; **137**: 791–7.

30 Garner JS, Jarvis WR, Emori TG *et al*. CDC definitions for nosocomial infections, 1988. *Am J Infect Control* 1988; **16**: 128–40.

31 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement M100-S17*. CLSI, Wayne, PA, USA, 2007.

32 Chung M, de Lencastre H, Matthews P *et al*. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist* 2000; **6**: 189–98.

33 Tenover FC, Arbeit RD, Goering RV *et al*. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.

34 Enright MC, Day NP, Davies CE *et al*. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; **38**: 1008–15.

35 Milheirico C, Oliveira DC, de Lencastre H. Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2007; **51**: 3374–7.

36 Shopsin B, Mathema B, Alcabes P *et al*. Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J Clin Microbiol* 2003; **41**: 456–9.

37 Lina G, Piemont Y, Godail-Gamot F *et al*. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 1999; **29**: 1128–32.

38 Gasch O, Ayats J, Angeles DM *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.

39 Cosgrove SE, Sakoulas G, Perencevich EN *et al*. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clin Infect Dis* 2003; **36**: 53–9.

40 Mylotte JM, Tayara A. *Staphylococcus aureus* bacteremia: predictors of 30-day mortality in a large cohort. *Clin Infect Dis* 2000; **31**: 1170–4.

41 Fang CT, Shau WY, Hsueh PR *et al*. Early empirical glycopeptide therapy for patients with methicillin-resistant *Staphylococcus aureus* bacteraemia: impact on the outcome. *J Antimicrob Chemother* 2006; **57**: 511–9.

42 Libert M, Elkholti M, Massaut J *et al*. Risk factors for meticillin resistance and outcome of *Staphylococcus aureus* bloodstream infection in a Belgian university hospital. *J Hosp Infect* 2008; **68**: 17–24.

43 Marchaim D, Kaye KS, Fowler VG *et al*. Case-control study to identify factors associated with mortality among patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. *Clin Microbiol Infect* 2010; **16**: 747–52.

44 Manzur A, Vidal M, Pujol M *et al*. Predictive factors of meticillin resistance among patients with *Staphylococcus aureus* bloodstream infections at hospital admission. *J Hosp Infect* 2007; **66**: 135–41.

45 Tacconelli E, Venkataraman L, De Girolami PC *et al*. Methicillin-resistant *Staphylococcus aureus* bacteraemia diagnosed at hospital admission: distinguishing between community-acquired versus healthcare-associated strains. *J Antimicrob Chemother* 2004; **53**: 474–9.

46 McGregor JC, Rich SE, Harris AD *et al*. A systematic review of the methods used to assess the association between appropriate antibiotic therapy and mortality in bacteremic patients. *Clin Infect Dis* 2007; **45**: 329–37.

47 Moellering RC Jr. What is inadequate antibacterial therapy? *Clin Infect Dis* 2009; **49**: 1006–8.

ORIGINAL ARTICLE 10.1111/1469-0691.12330

Lack of association between genotypes and haematogenous seeding infections in a large cohort of patients with methicillin-resistant *Staphylococcus aureus* bacteraemia from 21 Spanish hospitals

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Abstract

There is increasing concern regarding the association between certain methicillin-resistant *Staphylococcus aureus* (MRSA) genotypes and poor clinical outcome. To assess this issue, a large cohort of 579 subjects with MRSA bacteraemia was prospectively followed from June 2008 to December 2009, in 21 hospitals in Spain. Epidemiology, clinical data, therapy, and outcome were recorded. All MRSA strains were analysed in a central laboratory. Presence of a haematogenous seeding infection was the dependent variable in an adjusted logistic regression model. Of the 579 patients included in the study, 84 (15%) had haematogenous seeding infections. Microdilution vancomycin median MIC (IQR) was 0.73 (0.38–3) mg/L. Most MRSA isolates (n = 371; 67%) belonged to Clonal Complex 5 (CC5) and carried an SCCmec element type IV and agr type 2. Isolates belonging to ST8-agr1-SCCmecIV, ST22-agr1-SCCmecIV and ST228-agr2-SCCmecI—a single locus variant of ST5—accounted for 8%, 9% and 9% of the isolates, respectively. After adjusting by clinical variables, any of the clones was associated with increased risk of haematogenous seeding infections. Higher vancomycin MIC was not identified as an independent risk factor, either. In contrast, persistent bacteraemia (OR 4.2; 2.3–7.8) and non-nosocomial acquisition (3.0; 1.7–5.6) were associated with increased risk.

Keywords: Bloodstream infection, complicated bacteraemia, genotype, methicillin-resistant *Staphylococcus aureus*
Original Submission: 22 May 2013; **Revised Submission:** 2 July 2013; **Accepted:** 4 July 2013
Editor: G. Lina

Clin Microbiol Infect

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Background

Staphylococcus aureus is one of the microorganisms most frequently involved in bacteraemia worldwide. Compared with other microorganisms, it is associated with higher rates of morbidity and mortality [1].

Haematogenous seeding infections are a frequent feature of *S. aureus* bacteraemia [2]. This clinical scenario is observed in approximately 30–40% of episodes and can be predicted by the presence of factors such as community acquisition, unknown source, suggestive skin lesions, persistent fever and positive follow-up blood cultures [3].

Infective endocarditis (IE) caused by *S. aureus* is probably the most relevant complication due to this microorganism [4]. Notably, with mortality rates between 40% and 50%, methicillin-resistant *S. aureus* (MRSA) IE is associated with worse prognosis than methicillin-sensitive *S. aureus* IE [5].

Higher vancomycin minimum inhibitory concentration (V-MIC), has been described as an independent predictor of treatment failure [6] and complicated bacteraemia [7]. Given that most clinical studies do not include molecular microbiology assessment, it is not known whether certain MRSA genotypes are associated with worse outcome and enhanced risk of complications. However, a few studies have taken into account strain clonality in their analyses of outcome predictors with contradictory results [8–11]. In this study we evaluated the influence of MRSA genotypes on the appearance of haematogenous seeding infections in a large cohort of bacteraemia episodes.

Methods

Study period and patients

The study was conducted from June 2008 to December 2009 in 21 hospitals in Spain. Four hospitals had <500 beds; nine had 500–1000 beds; and eight had >1000 beds. An infectious disease specialist prospectively followed up adult patients (>16 years old) with MRSA blood cultures previously detected at the microbiology laboratory, and excluded those that did not meet the inclusion criteria (lack of signs and symptoms consistent with sepsis). A standardized protocol with demographic and clinical information was applied. Strains were sent to a central laboratory for further studies. The first isolate of each episode was used for the analysis.

Study design

With the data collected, MRSA bacteraemia episodes with and without observed haematogenous seeding infections were compared. To analyse whether there is an association with genetic background, we carried out an adjusted multivariate analysis in which presence of haematogenous seeding infection was the dependent variable and MRSA clonal complex was the independent variable of interest.

Definitions

The MRSA bacteraemia was defined as the presence of at least one positive blood culture for MRSA in a sample from a patient with clinical findings consistent with infection. When a focal infection different from bacteraemia portal of entry was diagnosed, the episode was considered a haematogenous seeding infection. Clinical data were interpreted accordingly with the infectious diseases specialist criteria.

Clinical variables. Co-morbidity was measured using the Charlson score, which stratifies the associated diseases on an ordinal scale. Three acquisition categories were considered: (i) nosocomial bacteraemia was considered when the episode was diagnosed at least 48 h after hospital admission, either to the ICU or to a conventional ward (non-ICU), and when there were no signs or symptoms of infection at admission; (ii) healthcare-related bacteraemia was diagnosed following Friedman's criteria; and (iii) community acquisition was considered when MRSA bacteraemia was diagnosed within 48 h of admission and when no previous contact with the healthcare system was recorded. The bacteraemia portal of entry was defined following CDC criteria [12]. Endocarditis was diagnosed in cases that met modified Duke criteria [13]. The severity of sepsis was determined on the basis of the Pitt score. Initial antibiotic was defined as the antibiotics administered in the first 48 h after bacteraemia onset. Definitive treatment was considered the antimicrobials administered after performing microbiological sensitivity tests. Antibiotic treatment was considered appropriate when the MRSA isolate was susceptible to at least one of the antibiotics administered, following the current CLSI breakpoints, with the exception of aminoglycosides, which were considered inappropriate, regardless of the sensitivity tests. Early intervention was considered when the catheter or foreign body was removed, or when a surgical procedure or drainage of infected source was performed within the first 48 h. Persistent bacteraemia was diagnosed when MRSA was isolated in blood cultures more than 48 h after the first dose of appropriate antibiotic. Relapse was diagnosed within 4 weeks after the end of therapy.

Susceptibility testing and molecular epidemiology of MRSA isolates

Each hospital identified the isolates and performed preliminary susceptibility tests. Further antimicrobial susceptibility testing

of all MRSA isolates was carried out in a central laboratory using the microdilution method. Commercial panels were used (ESTEN 2009, Sensititre; Izasa, Barcelona, Spain), and results were interpreted following the guidelines of the National Committee for Clinical Laboratory Standards (currently the CLSI).

Pulsed-field gel electrophoresis (PFGE) was performed after *Sma*I restriction of chromosomal DNA in all isolates, following a previously published method [14]. Restriction patterns were interpreted following the criteria published elsewhere [15]. To determine the multilocus sequence type (MLST) [16] and the staphylococcal chromosome cassette *mec* (SCC*mec*) types [17], we studied representative isolates of each PFGE type and subtype. Accordingly, MLSTs and SCC*mec* types were further inferred for all the strains. MLST sequences types (ST) were assigned through the MLST database (http://www.mlst.net), and clonal complexes (CC) were defined using the eBURST v3 algorithm (http://eburst.mlst.net). The *agr* polymorphism and the presence of genes in all the isolates were examined by PCR, following previously described methodology [18].

Statistical analysis

Qualitative and stratified continuous variables were compared using Fisher's exact test or Pearson's chi-squared test. Relative risks were calculated with 95% CI in a univariate analysis. Multivariate analysis was performed by using a logistic regression model in which presence of haematogenous seeding infection was the dependent variable. All the variables with theoretical clinical significance and those that achieved a p value <0.10 in the univariate analysis were included in the multivariate analysis, and adjusted OR were calculated with 95% CI. All analyses were performed using SPSS.v21 (Microsoft, Chicago, IL, USA).

Ethical considerations

The study was approved by the Spanish Network for Research in Infectious Diseases (REIPI) as well as the Institutional Review Board of each participating centre. Because no direct patient contact was planned, the requirement for informed consent was waived. The data were de-identified in each centre and only then transferred for analysis.

Results

Overall, 579 episodes of MRSA bacteraemia were included in the analysis. Their main characteristics are summarized in Table 1. Of these, 84 (15%) were diagnosed with haematogenous seeding infections. Twenty-five PFGE types were found among the 579 isolates (Fig. 1) [19]. A dominant PFGE genotype (pulse-type 2) was found in 371 isolates (67%), all of which belonged to CC5 (ST125 and ST146). They carried an SCC*mec* element type IV and *agr* type 2. Forty-seven (9%) isolates of clonal type ST228-agr2, a single locus variant of ST5, were considered a separate clone based on the SCC*mec* polymorphism type I. CC22-SCCmecIV-*agr*1 and CC8-SCC*mec*IV-*agr*1 accounted for 9% and 8%, respectively, of all the strains studied. CC5, CC228 and non-CC5/CC8/CC22/ST228 microdilution V-MIC_{50} and V-MIC_{90} were 0.75 and 1.0 mg/L, whereas those of CC22 and CC8 were 0.75 mg/L.

TABLE 1. Summary of episodes of methicillin-resistant *Staphylococcus aureus* bacteraemia

Clinical characteristics (n = 579)	***n* (%)**
Age	
Mean (SD)	69.1 (14.3)
Gender	
Female	194 (34)
Charlson	
Median (ICR)	3 (2–6)
McCabe	
Non-fatal	279 (49)
Ultimately fatal	212 (37)
Rapidly fatal	82 (14)
Portal of entry	
Vascular catheter	218 (38)
Unknown	123 (21)
Skin and soft tissues	81 (14)
Lower respiratory tract	70 (12)
Surgical site infections	36 (6)
Urinary tract	28 (5)
Haematogenous seeding infection	84 (15)
Endocarditis	32
Bone and joint infection	28
Lung	6
Endophthalmitis	5
Spleen	5
Central nervous system	4
Liver	1
Other	9
Persistent bacteraemia	93 (23)
Foreign body presence	270 (47)
Acquisition	
Nosocomial	338 (59)
Healthcare-related	238 (38)
Community	20 (4)
Pitt score	
Median (ICR)	1 (0–3)
Microbiological studies (n = 552)	
agr type	
II	419 (76)
I	122 (22)
III	11 (2)
MLST-SCCmec	
CC5[a]—IV	371 (67)
CC22—IV	48 (9)
ST228—I	47 (9)
CC8—IV	44 (8)
Other	42 (8)
Microdilution vancomycin median MIC (IQR) (mg/L)	0.73 (0.38–3)
Initial treatment	
Appropriate initial antibiotic	371 (66)
Source drainage or catheter withdrawal	205 (35)

MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; SCCmec, staphylococcal cassette chromosome; IQR, interquartile range.
[a]CC5 includes ST125 and ST146.

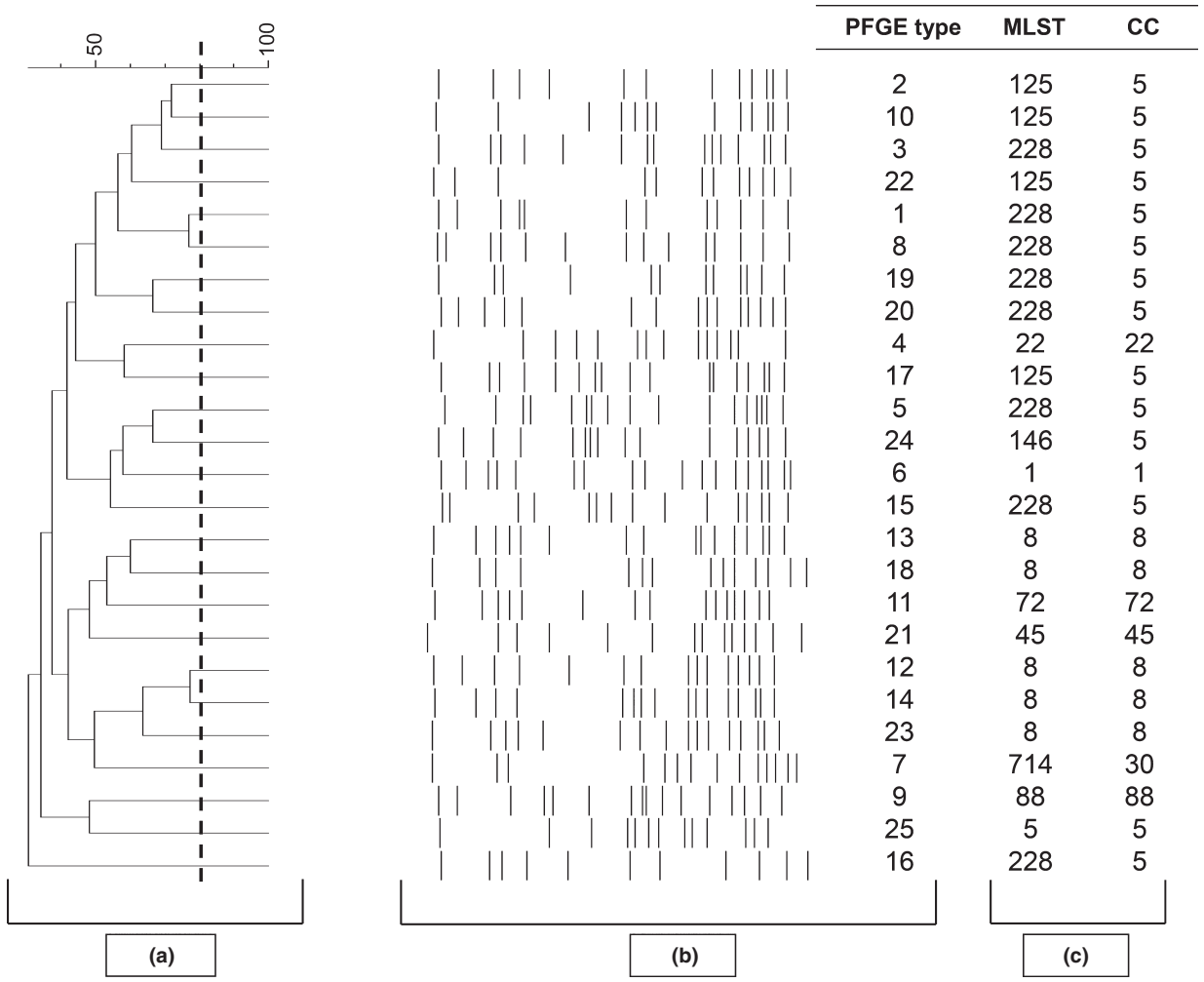

FIG. 1. Molecular characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. Shown from left to right are (a) a dendrogram comparing pulsed-field gel electrophoresis (PFGE) of *Sma*I macrorestriction fragments, (b) PFGE patterns and types (expressed by numbers), followed by (c) multilocus sequence typing (MLST) and clonal complex (CC). The cut-off value to define PFGE types was set at 80%. Optimization and band position tolerance were both set at 0.6%.

Impact of clonality on the appearance of haematogenous seeding infections

Haematogenous seeding infections were observed in 17% of the episodes caused by strains belonging to CC22, and in 13–15% of the episodes caused by strains belonging to the other genotypes (ST228, CC8, CC5 and other clones grouped).

The following clinical factors were used to adjust the logistic regression model: age ≤70 years, portal of entry, foreign body presence, acquisition, persistent bacteraemia, early intervention on portal of entry and appropriate initial antibiotic therapy. Episodes with and without haematogenous seeding infections had similar proportions of isolates with V-MIC > 1.5 mg/L in the univariate analysis (p 0.74) (Table 2). After adjusting by the former variables, any of the clones was associated with the presence of haematogenous seeding infections. In contrast, only persistent bacteraemia (OR 4.2; 2.3–7.8) and non-nosocomial acquired episodes (3.0; 1.7–5.6) were associated with an increased risk of haematogenous seeding infections (Table 3). Notably, there were no differences between the most prevalent clones, regarding the proportion of persistent bacteraemia episodes (31% of ST228; 28% of ST146; 23% of ST 125; 28% of ST22; 18% of ST8), nor between typical community clones grouped (ST8/USA300 and ST398; $n = 15$) and the rest of the clones (p 0.4). Lastly, persistence was also similar among the nosocomial and non-nosocomial acquisition cohorts (24% and 21%, respectively) (p 0.4).

Discussion

Although definitions between studies differ widely, predictive factors for complications among *S. aureus* bacteraemia have

TABLE 2. Predictive factors for methicillin-resistant *Staphylococcus aureus* haematogenous seeding infections: univariate logistic regression analysis

	Haematogenous seeding, *n* = 84 (15) *n* (%)	Non-haematogenous seeding, *n* = 492 (85) *n* (%)	Univariate p value
Age			
<70 years	38 (45)	275 (56)	0.08
Gender			
Female	30 (36)	162 (33)	0.62
Charlson			
>5 points	19 (23)	144 (29)	0.20
McCabe			
Non-fatal	42 (51)	235 (48)	
Ultimately fatal	33 (40)	178 (37)	0.88
Rapidly fatal	8 (9)	74 (15)	0.22
Pitt score			
>3 points	15 (18)	102 (21)	0.52
Portal of entry			
Intravascular catheter	24 (29)	193 (39)	
Unknown	27 (32)	95 (19)	0.01
Surgical site	10 (12)	26(5)	0.01
Skin and soft tissues	18 (21)	62 (13)	0.01
Lower espiratory tract	1 (1)	69 (14)	0.04
Foreign body presence	44 (52)	223 (45)	0.23
Acquisition			
Non-nosocomial[a]	46 (56)	190 (39)	0.03
Persistent bacteraemia	30 (42)	63 (19)	0.001
Clonal complex			
CC22	8 (9)	39 (9)	
CC5	54 (67)	316 (68)	0.83
CC8	6 (8)	37 (8)	0.79
ST228	6 (8)	41 (9)	0.56
Other	7 (8)	30 (6)	0.82
Microdilution vancomycin MIC ≥ 1.5 mg/L	3 (4)	15 (3)	0.74
Early intervention[b]	37 (44)	167 (34)	0.07
Appropriate initial antibiotic therapy	62 (74)	307 (63)	0.04

[a]Healthcare-acquired and community-acquired episodes are analysed together.
[b]Early intervention included catheter withdrawal, drainage of source or surgery within the first 48 h after MRSA bacteraemia onset.

TABLE 3. Adjusted logistic regression analysis for presence of haematogenous seeding infections among methicillin-resistant *Staphylococcus aureus* bacteraemia[a]

	Adjusted OR (95% CI)
Clonal complex	
CC22	
CC5[b]	2.3 (0.7–7.5)
CC8	2.1 (0.5–9.6)
ST228	1.2 (0.2–5.7)
Other clones	3.4 (0.7–15.6)
Persistent bacteraemia	4.2 (2.3–7.8)
Non-nosocomial acquisition	3.0 (1.7–5.6)

[a]Adjusted by age, portal of entry, foreign body presence, acquisition, persistent bacteraemia, early intervention on portal of entry and appropriate initial antibiotic therapy.
[b]CC5 includes ST125 and ST146.

been described. As in our study, persistent bacteraemia and community acquisition are some of the most relevant identified risk factors [2,3,20–24]. Surprisingly, the impact of distinctive clones on the appearance of complications has usually received little attention until now.

Taking advantage of a large well-characterized MRSA clinical series from bacteraemia episodes, the present study sought to determine whether there was an independent association between certain genotypes and the appearance of haematogenous seeding infections. Although this question has been addressed previously, inconsistent findings have been reported.

Although some studies did not find that MRSA genotypes differ in their ability to cause invasive infections, others did [25], thereby indicating a possible association between certain MRSA clones and some virulence factors. Furthermore, the influence of some *S. aureus* clonal groups on clinical outcome has also been assessed. Fowler *et al.* [9] recently found an association between CC5 and CC30, and haematogenous complications. In a subsequent study, they identified 14 non-SCC*mec*-specific genes that were associated with complicated bacteraemia [26]. Also, Wertheim *et al.* [27] observed higher mortality in episodes of bacteraemia caused by certain *S. aureus* CCs, and Seidl *et al.* [28] reported lower frequencies of persistent MRSA bacteraemia in episodes caused by CC1. It should be noted that all the former studies emphasized that all the genotypes examined could cause invasive disease.

The scientific community is increasingly concerned about the impact of higher V-MIC on outcome. In our study, loss of vancomycin susceptibility was not identified as a risk factor for haematogenous seeding infection. In this regard, two recent clinical studies have demonstrated an association between higher V-MIC and mortality [29], or complications [7] in episodes of *S. aureus* bacteraemia that were not treated with vancomycin. Those results revealed V-MIC to be a marker of certain specific strain factors, which might be related to a poorer outcome. To ascertain the effect of clonality on these associations, molecular characterization of strains would have been needed. However, it was not performed in those studies. In this regard, Miller *et al.* [11] reported a strong association between CC22 isolates with high V-MIC and endocarditis or septic metastasis, among catheter-related MRSA bacteraemias.

In contrast with the former studies, in our cohort of patients with MRSA bacteraemia, risk of haematogenous seeding infections was not associated with any of the analysed genotypes. A recent study of 159 patients with MRSA bacteraemia did not find an association between the risk of persistent bacteraemia and their clonal complexes (CC5, CC8 and CC15), either [8]. Therefore, the findings from these two studies would suggest that risk for complicated bacteraemia would be influenced by factors that depend on the host and the host's interaction with the microorganism rather than by the microorganism-specific characteristics.

All of these distinct results between studies might have different plausible explanations. First, the different definitions

used to assess outcomes. Second, the specific distribution of clones and virulence genes in each region may influence the existence of differences regarding their ability to cause complications [10]. For instance, our study registered a low presence of typical community clones (i.e. ST8 harbouring PVL, ST398), whereas the prevalence of these clones in other regions is higher [30]. In contrast, most of the isolates in our study belonged to CC5. Therefore, we cannot rule out the possibility that some of the less prevalent clones in our cohort may be associated with haematogenous seeding infections. Third, we must also consider the possibility of a publication bias related to the inclination to report positive results. Last, instead of genotypes, certain virulence factors more frequently harboured by some clones might be the authentic prognostic factors that influence the final outcomes. As a result, more clinical studies will be required to analyse differences between clones regarding their ability to increase the risk for worse outcomes, and specifically, to cause haematogenous seeding infections.

Acknowledgements

OG received a Río Hortega grant (CM08/228) from the Instituto de Salud Carlos III. This study was supported by the Ministerio de Ciencia e Innovación and the Instituto de Salud Carlos III (FIS 08/0335), and was co-financed by the European Regional Development Fund project 'A way to achieve Europe', undertaken by the Spanish Network for the Research in Infectious Diseases (REIPI RD06/0008).

Authors' Contributions

OG participated in the design of the study, contributed to the acquisition of data and performed the statistical analysis. He also participated in drafting the manuscript. MC and MAD carried out the microbiological studies including the molecular genetic studies and susceptibility tests. They also contributed to the final revision of the manuscript. BP, VP, BA, CM, FLM, ERG, JRB, GGP, EC, JH, AG, AJ and CD contributed to the acquisition of data and were involved in drafting and reviewing the manuscript. MP led and coordinated the study. He participated in the acquisition of data and revision of the final manuscript. All authors read and approved the final manuscript.

Transparency Declaration

BA received funding for research from Pfizer, Novartis, Gilead and MSD and funds for advisory board membership from Pfizer, Gilead, Novartis, Janssen, Astellas and MSD. JRB has received payments for lectures from Abbott Laboratories, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, Janssen, Merck and ViiV Healthcare. EC has received payments for lectures from Pfizer and travel expenses from Pfizer, MSD, Novartis and Astellas JPH has served as speaker for Pfizer, Astellas, Astra-Zeneca, Novartis and MSD. The rest of the authors have no conflicts of interest to declare.

References

1. Laupland KB, Gregson DB, Zygun DA, Doig CJ, Mortis G, Church DL. Severe bloodstream infections: a population-based assessment. *Crit Care Med* 2004; 32: 992–997.
2. Khatib R, Johnson LB, Fakih MG *et al.* Persistence in *Staphylococcus aureus* bacteremia: incidence, characteristics of patients and outcome. *Scand J Infect Dis* 2006; 38: 7–14.
3. Fowler VG Jr, Olsen MK, Corey GR *et al.* Clinical identifiers of complicated *Staphylococcus aureus* bacteremia. *Arch Intern Med* 2003; 163: 2066–2072.
4. Fowler VG Jr, Miro JM, Hoen B *et al. Staphylococcus aureus* endocarditis: a consequence of medical progress. *JAMA* 2005; 293: 3012–3021.
5. Miro JM, Anguera I, Cabell CH *et al. Staphylococcus aureus* native valve infective endocarditis: report of 566 episodes from the International Collaboration on Endocarditis Merged Database. *Clin Infect Dis* 2005; 41: 507–514.
6. Moise PA, Sakoulas G, Forrest A, Schentag JJ. Vancomycin *in vitro* bactericidal activity and its relationship to efficacy in clearance of methicillin-resistant *Staphylococcus aureus* bacteremia. *Antimicrob Agents Chemother* 2007; 51: 2582–2586.
7. Aguado JM, San-Juan R, Lalueza A *et al.* High vancomycin MIC and complicated methicillin-susceptible *Staphylococcus aureus* bacteremia. *Emerg Infect Dis* 2011; 17: 1099–1102.
8. Chong YP, Park SJ, Kim HS *et al.* Persistent *Staphylococcus aureus* bacteremia: a prospective analysis of risk factors, outcomes, and microbiologic and genotypic characteristics of isolates. *Medicine (Baltimore)* 2013; 92: 98–108.
9. Fowler VG Jr, Nelson CL, McIntyre LM *et al.* Potential associations between hematogenous complications and bacterial genotype in *Staphylococcus aureus* infection. *J Infect Dis* 2007; 196: 738–747.
10. Lalani T, Federspiel JJ, Boucher HW *et al.* Associations between the genotypes of *Staphylococcus aureus* bloodstream isolates and clinical characteristics and outcomes of bacteremic patients. *J Clin Microbiol* 2008; 46: 2890–2896.
11. Miller CE, Batra R, Cooper BS *et al.* An association between bacterial genotype combined with a high-vancomycin minimum inhibitory concentration and risk of endocarditis in methicillin-resistant *Staphylococcus aureus* bloodstream infection. *Clin Infect Dis* 2012; 54: 591–600.
12. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections, 1988. *Am J Infect Control* 1988; 16: 128–140.
13. Li JS, Sexton DJ, Mick N *et al.* Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. *Clin Infect Dis* 2000; 30: 633–638.
14. Chung M, deLencastre H, Matthews P *et al.* Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb Drug Resist* 2000; 6: 189–198.
15. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.

16. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; 38: 1008–1015.
17. Milheirico C, Oliveira DC, deLencastre H. Update to the multiplex PCR strategy for assignment of mec element types in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2007; 51: 3374–3377.
18. Shopsin B, Mathema B, Alcabes P *et al.* Prevalence of agr specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. *J Clin Microbiol* 2003; 41: 456–459.
19. Gasch O, Camoez M, Dominguez MA *et al.* Predictive factors for mortality in patients with methicillin-resistant *Staphylococcus aureus* bloodstream infection: impact on outcome of host, microorganism and therapy. *Clin Microbiol Infect* 2012; doi:10.1111/1469-0691.12108.
20. Chang FY, MacDonald BB, Peacock JE Jr *et al.* A prospective multicenter study of *Staphylococcus aureus* bacteremia: incidence of endocarditis, risk factors for mortality, and clinical impact of methicillin resistance. *Medicine (Baltimore)* 2003; 82: 322–332.
21. El-Ahdab F, Benjamin DK Jr, Wang A *et al.* Risk of endocarditis among patients with prosthetic valves and *Staphylococcus aureus* bacteremia. *Am J Med* 2005; 118: 225–229.
22. Fowler VG Jr, Justice A, Moore C *et al.* Risk factors for hematogenous complications of intravascular catheter-associated *Staphylococcus aureus* bacteremia. *Clin Infect Dis* 2005; 40: 695–703.
23. Hill EE, Vanderschueren S, Verhaegen J *et al.* Risk factors for infective endocarditis and outcome of patients with *Staphylococcus aureus* bacteremia. *Mayo Clin Proc* 2007; 82: 1165–1169.
24. Ringberg H, Thoren A, Lilja B. Metastatic complications of *Staphylococcus aureus* septicemia. To seek is to find. *Infection* 2000; 28: 132–136.
25. Feil EJ, Cooper JE, Grundmann H *et al.* How clonal is *Staphylococcus aureus*? *J Bacteriol* 2003; 185: 3307–3316.
26. Gill SR, McIntyre LM, Nelson CL *et al.* Potential associations between severity of infection and the presence of virulence-associated genes in clinical strains of *Staphylococcus aureus*. *PLoS ONE* 2011; 6: e18673.
27. Wertheim HF, van Leeuwen WB, Snijders S *et al.* Associations between *Staphylococcus aureus* genotype, infection, and in-hospital mortality: a nested case–control study. *J Infect Dis* 2005; 192: 1196–1200.
28. Seidl K, Bayer AS, Fowler VG Jr *et al.* Combinatorial phenotypic signatures distinguish persistent from resolving methicillin-resistant *Staphylococcus aureus* bacteremia isolates. *Antimicrob Agents Chemother* 2011; 55: 575–582.
29. Holmes NE, Turnidge JD, Munckhof WJ *et al.* Antibiotic choice may not explain poorer outcomes in patients with *Staphylococcus aureus* bacteremia and high vancomycin minimum inhibitory concentrations. *J Infect Dis* 2011; 204: 340–347.
30. Tristan A, Bes M, Meugnier H *et al.* Global distribution of Panton–Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. *Emerg Infect Dis* 2007; 13: 594–600.

Journal of Antimicrobial Chemotherapy Advance Access published October 9, 2013

Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother
doi:10.1093/jac/dkt396

Emergence of resistance to daptomycin in a cohort of patients with methicillin-resistant *Staphylococcus aureus* persistent bacteraemia treated with daptomycin

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Keywords: bloodstream infections, outcomes, MICs, cationic peptides

Sir,

Most published reports of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia treated with daptomycin have recorded high success rates.[1,2] However, therapeutic failure with emergence of resistance has also been observed, especially associated with deep-seated infections (e.g. endocarditis, osteomyelitis), high inocula of bacteria and low-dose regimens.[3] Although 6 mg/kg has usually been the standard recommended dose regimen, in recent Infectious Diseases Society of America (IDSA) guidelines 8–10 mg/kg regimens are considered for complicated bacteraemias and infective endocarditis (B-III).[4] We aimed to ascertain the clinical impact of susceptibility loss during daptomycin therapy in a cohort of patients with MRSA bacteraemia diagnosed and treated at 21 Spanish hospitals from June 2008 to December 2009. An infectious disease specialist prospectively followed the adult patients (>16 years old) with MRSA bacteraemia previously detected at the microbiology laboratory.[5] A standardized protocol with demographic and clinical information, including age, sex, morbidities, source and acquisition, diagnostic explorations, antibiotic treatment, follow-up and outcome, was applied. Extraction of blood cultures every 72 h until they were sterile was recommended in the protocol (though not mandatory for inclusion in the study). Episodes of documented bacteraemia for ≥7 days were classified as persistent bacteraemia. Daptomycin was the definitive therapy when it was administered immediately after obtaining antibiotic susceptibility information, either alone or combined with other antibiotic agents for at least 7 days, or otherwise until sterile blood cultures were obtained or the patient's death. For outcome, therapeutic failure included patients in which MRSA was still yielded by blood cultures despite daptomycin therapy (microbiological failure) as well as those receiving daptomycin as definitive therapy who died within 30 days of bacteraemia onset. Isolates were sent to a central reference laboratory. Susceptibility testing and molecular identification of all MRSA isolates were performed as published previously.[5] Associations between different variables and final outcomes (microbiological and clinical failure) were assessed using two-tailed Fisher's exact test. Differences were considered to be statistically significant when *P* values were ≤0.05. SPSS v15 was used for all the analyses. The study was approved by the Spanish Network for Research in Infectious Diseases (REIPI) as well as the Institutional Review Board of each participating centre. Because no direct patient contact was planned, the requirement for informed consent was waived.

Among 579 episodes of MRSA bacteraemia included in our study, daptomycin was used as initial therapy in 44/518 (8%) patients, while 124/518 (24%) patients received the antibiotic as definitive therapy: 77 (65%) at 6 mg/kg and 41 (35%) at higher doses (NB - in 6 patients we did not know the exact doses of daptomycin used). Concomitantly with daptomycin, 13 (10%) patients received other antibiotics with anti-staphylococcal activity: rifampicin ($n=6$), aminoglycosides ($n=3$), vancomycin, linezolid, teicoplanin and rifampicin plus gentamicin ($n=1$, each). Persistent bacteraemia was diagnosed in 22 of 124 (18%) episodes treated with daptomycin. Their main characteristics are summarized in Table 1. Significant increases in daptomycin MIC were observed in subsequent isolates of 7 of 18 (39%) episodes. Death within 30 days was observed in 7 of 22 (32%) and microbiological failure in 9 of 22 (41%) episodes. Daptomycin MIC increase was significantly associated with microbiological failure [OR 27 (1.9–368.4)] and with therapeutic failure of any cause [OR 16 (1.3–194.6)]. By contrast, none of the other factors evaluated was associated.

Few clinical reports of daptomycin therapeutic failures with emergence of resistance have been published until now; most of the patients were treated with low doses of daptomycin or had deep infections associated with high inocula of bacteria.[6] Two of these studies demonstrated daptomycin susceptibility loss. First, in a clinical trial that compared daptomycin (6 mg/kg) with standard therapy against *S. aureus* bacteraemia, isolates with reduced susceptibility to daptomycin emerged in 6 of the 19 episodes (32%) with persistent or relapsing bacteraemia.[7] Secondly, in a subcohort of 10 episodes of *S. aureus* persistent bacteraemia

Table 1. MRSA persistent bacteraemia episodes treated with daptomycin

	Patient			MRSA isolates		Therapy			Outcome	
ID	age (years)/ gender	previous VAN	source/acquisition	initial/ final DAP MIC (mg/L)	initial/ final VAN MIC (mg/L)	initial therapy	DAP[a], regimen dose (days from first dose to last blood culture yielding MRSA)	intervention on source (days from DAP first dose)	sterile blood culture	30 day mortality
1	84/M	no	catheter/HCR	**≤0.5/2**	0.5/1	DAP	10 mg/kg (18)	catheter withdrawal (−7)	no	yes
2	83/M	no	IE/HCR	**≤0.5/2**	**0.7/1.5**	DAP	10 mg/kg (13)	no	no	yes
3	76/F	no	catheter/ICU	**≤0.5/1.5**	0.5/1	VAN	10 mg/kg (13)	catheter withdrawal (−2)	no	yes
4	84/M	no	catheter/non-ICU	≤0.5/1	0.5/0.5	VAN	8 mg/kg (7)	catheter withdrawal (−1)	no	yes
5	58/M	no	SSTs/non-ICU	≤0.5/NA	1/NA	VAN	10 mg/kg (7)	no	no	yes
6	82/F	no	IE/HCR	≤0.5/≤0.5	0.5/0.5	LZD	8 mg/kg (9)+RIF	surgery (−3)	no	yes
7	41/M	no	catheter/non-ICU	≤0.5/≤0.5	1/1	VAN	8 mg/kg (9)	catheter withdrawal (−6)	yes, with DAP 8	yes
8	74/F	yes	bone and joint infection/HCR	**≤0.5/1.5**	0.5/0.5	DAP	10 mg/kg (28)	no	yes, with FOS+IPM	no
9	65/M	no	catheter/ICU	**≤0.5/2**	0.5/1	VAN	10 mg/kg (21)	catheter withdrawal (−2)	yes, with FOS+IPM	no
10	78/F	no	unknown/non-ICU	**<0.5/2**	0.5/1	no antibiotic	8 mg/kg (21)	no	yes, with LZD	no
11	81/M	no	SSTs/non-ICU	≤0.5/NA	1/NA	DAP	6 mg/kg (6)	no	yes, with DAP 8	no
12	69/F	no	SSTs/HCR	≤0.5/≤0.5	1/1	VAN	10 mg/kg (5)	no	yes, with DAP 10	no
13	69/M	yes	SSTs/non-ICU	**0.75/3**	1.5/2	DAP	10 mg/kg (8)	drainage (0)	yes, with DAP 10	no
14	80/M	no	urinary/HCR	0.5/0.5	1/1	no antibiotic	6 mg/kg (4)	no	yes, with DAP 6	no
15	58/M	no	catheter/non-ICU	≤0.5/NA	1/NA	VAN	10 mg/kg (16)	catheter withdrawal (−1)	yes, with DAP 10	no
16	73/M	no	catheter/HCR	≤0.5/NA	1/NA	DAP	8 mg/kg (9)	catheter withdrawal (0)	yes, with DAP 8	no
17	52/M	yes	catheter/HCR	≤0.5/≤0.5	1/≤0.5	VAN	8 mg/kg (3)+RIF	catheter withdrawal (−1)	yes, with DAP 8+RIF	no
18	82/M	no	catheter/non-ICU	≤0.5/≤0.5	1/1	DAP	8 mg/kg (6)	catheter withdrawal (−3); surgery on metastatic infection (+14)	yes, with DAP 8	no
19	77/M	no	IE/HCR	≤0.5/1	**0.5/2**	VAN	6 mg/kg (21)+GEN+RIF	no	yes, with DAP 6+GEN	no
20	70/M	no	catheter/HCR	≤0.5/≤0.5	2/0.5	DAP	6 mg/kg (5)	catheter withdrawal (+8)	yes, with DAP 6	no
21	66/M	no	unknown/non-ICU	≤0.5/≤0.5	0.5/0.5	DAP	6 mg/kg (11)	no	yes, with DAP 6	no
22	60/M	no	unknown/HCR	≤0.5/≤0.5	0.5/0.5	VAN	8 mg/kg (27)	surgery (+3)	yes, with DAP 8	no

M, male; F, female; HCR, healthcare related; ICU, intensive care unit; IE, infective endocarditis; SSTs, skin and soft tissues; NA, data not available; DAP, daptomycin; FOS, fosfomycin; GEN, gentamicin; IPM, imipenem; LZD, linezolid; RIF, rifampicin; VAN, vancomycin.
Episodes 1–10 had microbiological and/or clinical failure (microbiological failure, patients 1–6 and 8–10; clinical failure, patients 1–7).
Bold numbers in MRSA isolates columns show significant changes in the antibiotic MIC.
MICs were determined using the microdilution method.
[a]Definitive therapy.

treated with daptomycin at 4–6 mg/kg, subsequent isolates from 6 (60%) episodes showed increased daptomycin MIC.[8] To our knowledge, our study is the first that includes a high proportion of patients with persistent MRSA bacteraemia treated with high-dose regimens. Importantly, the rate of emergence of resistance was apparently similar to that observed in the clinical trial cited above, in which daptomycin therapy was administered in low-dose regimens.[7] However, since we only included episodes with persistent bacteraemia for $\geq$7 days, we must stress the existence of selection bias with respect to this other study. Despite the limitations related to the low number of episodes included, the present study provides valuable data regarding the incidence of daptomycin susceptibility loss during persistent MRSA bacteraemia and suggests an association of this phenomenon with subsequent therapeutic failure. As a consequence, our results reinforce the recommendation of monitoring daptomycin susceptibility in subsequent isolates to assess the risk of final therapeutic failure.

Acknowledgements

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Funding

This work was supported by the Ministerio de Ciencia e Innovación and the Instituto de Salud Carlos III (FIS 08/0335), and co-financed by the European Regional Development Fund project 'A way to achieve Europe', undertaken by the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008). O. G. received a Río Hortega grant (grant number CM08/228) from the Instituto de Salud Carlos III. F. L.-M. is partially supported by a grant from the Research Intensification Programme in the National Health Care System (grant number I3SNS) from the Spanish Ministry of Economy and Competitiveness, Instituto de Salud Carlos III.

Transparency declarations

B. A. has received funding for research from Pfizer, Novartis, Gilead and MSD, and funds for advisory board membership from Pfizer, Gilead, Novartis, Janssen, Astellas and MSD. J. R. B. has received payments for lectures from Abbott Laboratories, Boehringer-Ingelheim, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, Janssen, Merck and ViiV Healthcare. E. C. has received payments for lectures from Pfizer and travel expenses from Pfizer, MSD, Novartis and Astellas. All other authors have no conflicts of interest to declare.

References

1 Moore CL, Osaki-Kiyan P, Haque NZ *et al*. Daptomycin versus vancomycin for bloodstream infections due to methicillin-resistant *Staphylococcus aureus* with a high vancomycin minimum inhibitory concentration: a case–control study. *Clin Infect Dis* 2012; **54**: 51–8.

2 Falagas ME, Giannopoulou KP, Ntziora F *et al*. Daptomycin for endocarditis and/or bacteraemia: a systematic review of the experimental and clinical evidence. *J Antimicrob Chemother* 2007; **60**: 7–19.

3 Moise PA, North D, Steenbergen JN *et al*. Susceptibility relationship between vancomycin and daptomycin in *Staphylococcus aureus*: facts and assumptions. *Lancet Infect Dis* 2009; **9**: 617–24.

4 Liu C, Bayer A, Cosgrove SE *et al*. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. *Clin Infect Dis* 2011; **52**: e18–55.

5 Gasch O, Camoez M, Domínguez MA *et al*. Predictive factors for early mortality among patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. *J Antimicrob Chemother* 2013; **68**: 1423–30.

6 Bayer AS, Schneider T, Sahl HG. Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. *Ann N Y Acad Sci* 2013; **1277**: 139–58.

7 Fowler VG Jr, Boucher HW, Corey GR *et al*. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. *N Engl J Med* 2006; **355**: 653–65.

8 Sharma M, Riederer K, Chase P *et al*. High rate of decreasing daptomycin susceptibility during the treatment of persistent *Staphylococcus aureus* bacteremia. *Eur J Clin Microbiol Infect Dis* 2008; **27**: 433–7.

Imaz et al. BMC Infectious Diseases (2015) 15:243
DOI 10.1186/s12879-015-0991-z

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Prevalence of methicillin-resistant *Staphylococcus aureus* colonization in HIV-infected patients in Barcelona, Spain: a cross-sectional study

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Abstract

Background: Colonization by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has been found to be markedly more common in HIV-infected individuals in the USA. Studies evaluating the prevalence MRSA colonization in HIV-infected populations in Europe are scarce. The aim of this study was to investigate the prevalence of MRSA colonization in a cohort of HIV-infected patients in Barcelona, Spain.

Methods: Nasal and pharyngeal *S. aureus* carriage was assessed in a random sample of 190 patients from an outpatient HIV clinic. Nasal and pharyngeal swab specimens were obtained for staphylococcal culture from 190 and 110 patients respectively. All MRSA isolates were screened for Panton-Valentine leukocidin (PVL) genes by PCR. Molecular characterization of MRSA isolates was performed by multilocus sequence typing. Data related to HIV infection, healthcare exposure, and previously described risk factors for MRSA were collected from medical records and a questionnaire administered to each patient.

Results: The patients' characteristics were as follows: male, 83 %; median (IQR) age, 45 (39–49) years; intravenous drug users, 39 %; men who have sex with men, 32 %; heterosexual, 26 %; CD4 count, 528/μL (IQR 351–740); on antiretroviral therapy, 96 %; and undetectable plasma viral load, 80 %. Sixty-five patients (34 %) were colonized by *S. aureus*. MRSA colonization was found in 1 % and 2 % of nasal and pharyngeal samples respectively. No PVL positive MRSA strains were detected and all the MRSA isolates belonged to typical hospital-acquired clones.

Conclusions: Our data suggest that CA-MRSA colonization is not currently a problem in HIV-infected individuals in our area.

Keywords: HIV, Methicillin-resistant *Staphylococcus aureus*, Colonization

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a causal agent of infection in individuals without risk factors for healthcare- associated MRSA acquisition that has been described as community-associated MRSA (CA-MRSA) [1]. CA-MRSA infections are usually distinguishable from their healthcare-related counterparts by epidemiological, molecular, and clinical features [2]. The incidence of the former is markedly increased in certain population groups such as HIV-infected patients, who have also been noted to have a higher risk of CA-MRSA colonization [3, 4]. In addition, HIV-infected individuals are frequently exposed to healthcare environments, and related factors such as prior hospitalization have also been linked to a higher rate of MRSA colonization in this population [5]. Nasal and extranasal MRSA colonization has been associated with an increased risk of subsequent infections in both HIV-negative and HIV-infected individuals [6, 7]. Additionally, colonization might represent a reservoir for

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MRSA transmission. As HIV-infected patients are commonly exposed to healthcare settings, it is important to assess the prevalence of colonization to determine the need for preventive and control measures, at least in hospital settings.

Most studies showing a high prevalence of MRSA colonization among HIV-infected individuals have been conducted in the USA. However, studies in certain US regions [8] and Europe [5, 9–12] have reported low prevalence.

Few European studies have evaluated MRSA colonization or infection in the HIV-infected population [5]. The aim of this study was to assess the prevalence of MRSA colonization in a cohort of HIV-infected patients in Spain.

Methods

This is a cross-sectional study assessing *S. aureus* colonization in a random sample of 190 adults (>18 years) from a total of 1665 patients seen at an outpatient HIV clinic in Barcelona, Spain between June 2011 and June 2012. Patients were randomly selected using a statistical software package (IBM SPSS version 19.0, Chicago, IL). *S. aureus* colonization was investigated by taking nasal swabs from all 190 patients and pharyngeal swabs from 110 patients. Swabs were plated onto MRSA agar medium (MRSA Select; Bio-Rad Laboratories, Madrid, Spain) and coagulase-mannitol salt agar plates (BBL™ Coagulase Mannitol Agar; BD, Madrid, Spain), and also inoculated into staphylococcal enrichment broth (BBL brain-heart infusion; BD, plus 7 % NaCl). After 24 h of incubation at 35-37 °C, the broths were subcultured onto MRSA Select and coagulase-mannitol salt plates. All plates were incubated for 48 h and inspected daily for *S. aureus*–like colonies. *S. aureus* colonies were identified by latex agglutination (Pastorex Staph-plus; Bio-Rad Laboratories) and DNase production (DNase Test Agar; Difco, Fco. Soria Melguizo, Madrid, Spain). Antibiotic susceptibility testing, including cefoxitin, was performed by the disc-diffusion method following CLSI recommendations [13]. All MRSA isolates were screened for Panton-Valentine leukocidin (PVL) genes by PCR. Molecular characterization of MRSA isolates was performed by multilocus sequence typing and SCC*mec* characterization as described previously [14, 15].

HIV infection–related data and epidemiological characteristics previously described as community or healthcare-related risk factors for MRSA were collected from medical records and a questionnaire completed by each patient (Table 1) [1].

CA-MRSA infection was defined according to the US Centers for Disease Control and Prevention 2000 criteria: 1) Diagnosis of MRSA in an outpatient setting or by a culture positive for MRSA within 48 hours of admission to hospital; 2) No medical history of MRSA infection or colonization; and 3) No medical history in the past year of: a) hospitalization; admission to a nursing home, skilled nursing facility, or hospice; c) dialysis; d) surgery; 4) permanent indwelling catheters or medical devices that pass through the skin into the body [1]. Since MRSA clones identified as typically CA-MRSA have distinguishing genetic features from hospital-acquired strains, such as SCC*mec* types IV and V and PVL expression, molecular characterization was also used to ascertain the possible origin of the strains (community vs healthcare environment) [2].

Table 1 Patient characteristics

	(*n* = 190)
Age, y (median, IQR)	45 (39–49)
Sex (male/female)	158/32 (83 %/17 %)
HIV risk factor	74 (39 %)
IDU	61 (32 %)
MSM	49 (26 %)
Heterosexual	6 (3 %)
Other/unknown	
AIDS	55 (29 %)
HCV	61 (32)
HBV	8 (4 %)
Current ART	182 (96 %)
$CD4^+$ T cell count, cells/μL (median, IQR)	528 (351–740)
$CD4^+$ T cell count, <200 cells/μL	19 (10 %)
HIV RNA, <40 copies/mL	153 (80 %)
Current use of TMP-SMX	15 (8 %)
Origin	Delete
Spain	161 (85%)
Europe	1 (0.5%)
South America	19 (10%)
North Africa	4 (2%)
Sub-Saharan Africa	4 (2%)
Asia	1 (0.5%)
Risk factors for HA-MRSA acquisition	
Antibiotic use in prior 12 months	70 (37 %)
Hospitalization in prior 12 months	32 (17 %)
Intravenous catheter use in prior 12 months	40 (21 %)
Surgical intervention in prior 12 months	19 (10 %)
Frequent visits (≥1 per week) to long-term care facilities	17 (9 %)
Multiple sexual partners in prior 12 months	17 (9 %)
History of sexually transmitted infection	17 (9 %)
Previous incarceration	29 (15 %)

Data are shown as number (%) of patients unless otherwise indicated.
IDU, injecting drug user; IQR, interquartile range; HBV, hepatitis B virus; HCV, hepatitis C virus; MSM: men who have sex with men;
TMP-SMX: trimethoprim-sulfamethoxazole

Continuous variables are reported as medians and interquartile range and categorical variables as numbers and percentages. The former were compared using the non-parametric Mann–Whitney U test while the latter were compared using the X^2 or Fisher's exact test, with a significance level of 0.05 (two-sided). A multivariate logistic regression model including demographic characteristics (age, gender, origin, HIV acquisition route), CD4+ T cell count, viral load, use of ART, healthcare-related related risk conditions (recent hospitalization or admission to a nursing home, recent surgical intervention, recent use of antibiotics), and other epidemiologic conditions (history of incarceration, history of sexually transmitted infections, multiple sexual partners) was used to identify independent risk factors for *S. aureus* and CA-MRSA colonization.

The study protocol was approved by the Clinical Investigation Ethics Committee of Bellvitge University Hospital and all patients gave written informed consent prior to participation.

Results

The patients' characteristics are summarized in Table 1. There were no significant statistical differences between patients with nasal and pharyngeal assessment (n = 110) and those with nasal assessment only (n = 80) (data not shown).

MRSA colonization was detected in 3 patients. Nasal colonization was observed in 2/190 individuals (1 %) and pharyngeal colonization in 2/110 individuals (2 %). Among patients with nasal and pharyngeal samples available, MRSA was present in both nasal and pharyngeal samples in 1 subject whilst the other patient was a pharyngeal carrier exclusively. Among those patients with MRSA colonization, two of them had risk factors for nosocomial acquisition. The two isolates were identified as ST146 and ST125, part of Clonal Complex 5 (CC5), and they both carried SCC*mec* IV (Table 2), which are indistinguishable features from the dominant hospital-acquired MRSA clone complex in our area [16]. The isolate belonging to the third patient was not available for molecular studies but antibiotic susceptibility and PVL results were available. Although the patient had no apparent risk factors for hospital acquisition, the antibiotic resistance pattern (resistance to erythromycin and ciprofloxacin) was identical to that of the dominant hospital-acquired MRSA lineage (Table 2).

Colonization by methicillin-susceptible *S. aureus* (MSSA) was documented in 62/190 nasal samples (32.6 %) and 3/110 pharyngeal samples (2.7 %), with only 1 in 3 patients found to be an exclusive MSSA pharyngeal carrier. None of the risk factors analyzed, including those related to demography, HIV acquisition route, HIV control, healthcare-related conditions, and other epidemiologic conditions, was statistically associated with MSSA colonization in either the univariate or multivariate analyses.

Discussion

The prevalence of *S. aureus* colonization in our HIV-infected population is consistent with rates reported for the general population [17]. However, only 3 individuals (corresponding to 2/190 nasal samples and 2/110 pharyngeal samples) were colonized by MRSA and none of the isolates belonged to a typical CA-MRSA lineage. Our data contrast with most reports from the USA, where a significant association between HIV infection and MRSA colonization has been well documented [3–7]. A recent meta-analysis assessing MRSA colonization prevalence and risk factors in HIV-infected individuals reported that 6.9 % of the population studied were MRSA carriers and that this rate rose to 8.8 % when only studies from North America were included [5]. Although data on MRSA carriage in HIV-infected individuals in Europe are scarce, lower prevalence rates have been reported (0 % to 2.8 %) [5, 9–12], and the findings in our series are consistent with reports from other European studies.

Several US studies have described an association between CA-MRSA colonization and risk factors such as sexual behavior, substance abuse, incarceration, and area of residence [4, 6, 7]. These risk groups were represented in our sample (Table 1).

We recently reported a series of MRSA infection in HIV-infected patients in Spain in which we detected a higher risk for MRSA infection among individuals with poorly controlled HIV infection and immigrants (mostly South Americans) [18]. Nevertheless, the overall prevalence was still lower than that reported for the USA. The absence of CA-MRSA colonization in HIV-infected individuals in our area could partly explain the low rate of CA-MRSA infection in this population.

Table 2 Characterization of MRSA isolates

Patient	Sample	SCC*mec*	MLST	PVL	Antibiotic resistance pattern
A	Pharyngeal	Type IV	ST146 (CC5)	Negative	Resistance to: erythromycin, clindamycin, tobramycin and ciprofloxacin
B	Nasal and Pharyngeal	Type IV	ST125 (CC5)	Negative	Resistance to: erythromycin, tobramycin and ciprofloxacin
C	Nasal	NA	NA	Negative	Resistance to: erythromycin, tobramycin and ciprofloxacin

CC, clonal complex; MLST, multilocus sequence typing; PVL, Panton–Valentine Leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type

Our study is the first to assess the prevalence of MRSA colonization in HIV-infected patients in Spain. However, it has some limitations. Although our sample is representative of our cohort of HIV-infected patients, the number of individuals studied is lower than in other series. Additionally, while MRSA colonization of extranasal areas such as the buttocks and the perianal, inguinal and axillary regions has been well documented [4, 7, 19, 20], the only extranasal location studied in our case was the pharynx, and paired nasal and pharyngeal samples were only available for a subset of 110 patients. Thus, the rate of MRSA colonization in our cohort might have been underestimated. The importance of extranasal colonization has been demonstrated for both hospital- and community-acquired MRSA [4, 7, 19, 20]. Recent studies in the community setting have observed that nasal-only screening could miss up to 51 % of MRSA colonized individuals [4, 19]. In the subset of patients with both nasal and pharyngeal samples in our series, MRSA was detected in both samples in one patient while the other one was an exclusive pharyngeal carrier. None of the patients had exclusive nasal colonization in this subgroup. Although we do not discount the importance of extranasal colonization (the colonization rate would have been 50 % lower if our study had been limited to nasal samples), the rate of MRSA colonization in our cohort was low.

We were unable to genetically characterize one MRSA isolate. However, PVL expression, which is characteristic in CA-MRSA strains in our area, was not detected, and the antibiotic susceptibility pattern, while unspecific, was indistinguishable from the dominant hospital-acquired MRSA lineage. Thus, the molecular characterization results for the available isolates, the absence of PVL production, and the antibiotic resistance patterns detected suggested healthcare-related acquisition in all cases.

Conclusions

Although nasal and/or pharyngeal *S. aureus* colonization was observed in one-third of HIV-infected patients in our cohort, the prevalence of MRSA carriage was low and colonization by typical CA-MRSA strains was not observed. Thus, MRSA colonization in the HIV-infected population in our area does not seem to be an epidemiological problem requiring specific control strategies to prevent cross-transmission and infection among HIV-infected individuals. More studies are needed to assess MRSA colonization rates in different European HIV-infected populations.

Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

AI and DP initiated this project. OG and MP contributed to the design of the study. AI, MM, AV and DP were responsible for sample collection. MC and MAD performed all microbiological procedures. AI and SDY were responsible for data collection and statistical analysis. AI drafted the manuscript and SDY, MC, MAD, OG, MP and DP reviewed it. All authors revised the manuscript for important intellectual content and contributed to the final version.

Acknowledgments

This study would not have been possible without the collaboration of all the patients, physicians, and nurses who took part in the project.
We thank Anne Murray for her assistance in the English language reviewing and editing of the manuscript prior to submission.
Sources of financial support: This study was partially funded by the RD12/0017/0013 project within the Spanish Research, Development and Innovation Plan and by the Instituto de Salud Carlos III (Subdirección General de Evaluación) and the European Regional Development Fund.

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Received: 1 February 2015 Accepted: 19 June 2015
Published online: 26 June 2015

References

1. Naimi TS, LeDell KH, Como-Sabetti K, et al. Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. JAMA. 2003;290:2976–84.
2. 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.
3. Hidron AI, Kourbatova EV, Halvosa JS, et al. Risk factors for colonization with methicillin-resistant Staphylococcus aureus (MRSA) in patients admitted to an urban hospital: emergence of community-associated MRSA nasal carriage. Clin Infect Dis. 2005;41:159–66.
4. Popovich KJ, Hota B, Aroutcheva A, et al. Community-associated methicillin-resistant Staphylococcus aureus colonization burden in HIV-infected patients. Clin Infect Dis. 2013;56:1067–74.
5. Zervou FN, Zacharioudakis IM, Ziakas PD, Rich JD, Mylonakis E. Prevalence of and risk factors for methicillin-resistant Staphylococcus aureus colonization in HIV infection: a meta-analysis. E Clin Infect Dis. 2014;59:1302–11.
6. Shet A, Mathema B, Mediavilla JR, et al. Colonization and subsequent skin and soft tissue infection due to methicillin-resistant Staphylococcus aureus in a cohort of otherwise healthy adults infected with HIV type 1. J Infect Dis. 2009;200:88–93.
7. Szumowski JD, Wener KM, Gold HS, et al. Methicillin-resistant Staphylococcus aureus colonization, behavioral risk factors, and skin and soft-tissue infection at an ambulatory clinic serving a large population of HIV-infected men who have sex with men. Clin Infect Dis. 2009;49:118–21.
8. Madariaga MG, Ullrich F, Swindells S. Low prevalence of community-acquired methicillin-resistant Staphylococcus aureus colonization and apparent lack of correlation with sexual behavior among HIV-infected patients in Nebraska. Clin Infect Dis. 2009;48:1485–7.
9. Giuliani M, Longo B, Latini A, et al. No evidence of colonization with community-acquired methicillin-resistant Staphylococcus aureus in HIV-1-infected men who have sex with men. Epidemiol Infect. 2010;138:738–42.
10. Oliva A, Lichtner M, Mascellino MT, et al. Study of methicillin-resistant Staphylococcus aureus (MRSA) carriage in a population of HIV-negative migrants and HIV-infected patients attending an outpatient clinic in Rome. Ann Ig. 2013;25:99–107.
11. Seybold U, Supthut-Schroder B, Draenert R, Hogardt M, Bogner JR. Prevalence and risk factors of nasal colonization with Staphylococcus aureus – association with HIV infection in older patients. Scand J Infect Dis. 2009;41:63–6.
12. Joore IK, van Rooijen MS, Schim van der Loeff MF, de Neeling AJ, van Dam A, de Vries HJ. Low prevalence of methicillin-resistant Staphylococcus aureus among men who have sex with men attending an STI clinic in Amsterdam: a cross-sectional study. BMJ Open. 2013;3(3):pii:e002505.
13. CLSI (2010) Performance standards for antimicrobial susceptibility testing; 20th Informational Supplement. CLSI document M100-S20. Wayne, pa: Clinical and laboratory standards institute; 2010.

14. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. J Clin Microbiol. 2000;38(3):1008–15.
15. Oliveira DC, De Lencastre H. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2002;46:2155–61.
16. Gasch O, Camoez M, Dominguez MA, et al. Predictive factors for mortality in patients with methicillin-resistant *Staphylococcus aureus* bloodstream infection: impact on outcome of host, microorganism and therapy. Clin Microbiol Infect. 2013;19(11):1049–57.
17. Schechter-Perkins EM, Mitchell PM, Murray KA, Rubin-Smith JE, Weir S, Gupta K. Prevalence and predictors of nasal and extranasal staphylococcal colonization in patients presenting to the emergency department. Ann Emerg Med. 2011;57:492–9.
18. Imaz A, Cobos-Trigueros N, Falcó V. Community-associated methicillin-resistant Staphylococcus aureus infections in HIV-infected patients in Spain. J Infect. 2013;66:199–201.
19. Miller LG, Eells SJ, Taylor AR, et al. Staphylococcus aureus colonization among household contacts of patients with skin infections: risk factors, strain discordance, and complex ecology. Clin Infect Dis. 2012;54:1523–35.
20. McKinnell JA, Huang SS, Eells SJ, Cui E, Miller LG. Quantifying the impact of extranasal testing of body sites for methicillin-resistant Staphylococcus aureus colonization at the time of hospital or intensive care unit admission. Infect Control Hosp Epidemiol. 2013;34:161–70.

Papers at scientific conferences

a) International meetings

1. MA. Domínguez, M. Camoez, M. Pujol, V. Mick, A. Manzur, J. Liñares, R. Martín. Genetic characterization of *Staphylococcus aureus* isolates producing Panton-Valentine Leukocidin. Poster 1458. 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC09). San Francisco, 12-15 September 2009.

2. O. Gasch, A. Hornero, A. Fernandez, C. Suarez, S. Gomez, M. Camoez, MA. Domínguez, J. Liñares, J. Ariza, M. Pujol. Panton-Valentine Leukocidin-Positive Methicillin-Susceptible *Staphylococcus aureus* (PVL-MSSA) Outbreak in Barcelona Area, Spain. Poster K-1707. 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC10). Boston, 12-15 September 2010.

3. O.Gasch, M. Camoez, MA. Domínguez, B. Padilla, V. Pintado, B. Almirante, C. Martin, M. Lagarde, E. Ruiz, JA. Martínez, M. Montejo, C. Lara, A. Arnaiz, MA. Goenaga, N. Benito, J. Rodríguez-Baño, M. Pujol on behalf of REIPI/GEIH study groups. Predictive factors for early mortality (EM) in patients with Methicillin-resistant *Staphylococcus aureus* (MRSA) bloodstream infection (BSI). Prospective study in 22 Spanish hospitals. Poster K-292. 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC10). Boston, 12-15 September 2010.

4. JM Sierra, M. Camoez, MA. Dominguez, A. Fernandez, M. Pujol, R. Martín. Characterization of endemic Methicillin resistant *Staphylococcus aureus* (MRSA) strains analysed by DNA-Microarray Genotyping (DMG). Poster C2-1491. 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC10). Boston, 12-15 September 2010.

5. M. Camoez, MA. Domínguez, J. Ayats, F. Tubau, O. Gasch, M. Pujol, R. Martín, J. Liñares. Comparative In vitro activity of daptomycin and

vancomycin against methicillin resistant *Staphylococcus aureus* (MRSA) blood isolates representative of major Spanish clones. Poster E-1554. 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC10). Boston, 12-15 September 2010.

6. O. Gasch, M. Camoez, M. Dominguez, B. Padilla, V. Pintado, B. Almirante, C.Martin, F. Lopez-Medrano, E. Ruiz, J. Blanco, G. Garcia-Pardo, E. Calbo, M. Montero, J. Horcajada, A. Granados, A. Jover, C. Dueñas, M. Pujol. Predictive Factors (PF) for Persistence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Bloodstream nfection (BSI). Prospective Study in 22 Spanish Hospitals. 51th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC11). Chicago, 17-20 September 2011.

7. JM. Sierra, M. Camoez, M. Pujol, R. Martin, MA. Domínguez. Biofilm formation of Different Lineages of Methicillin Resistant Staphylococcus aureus (MRSA). 51th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC11). Chicago, 17-20 September 2011.

8. O. Gasch, M. Camoez, M. Domínguez, B. Padilla, V. Pintado, B. Almirante, C.Martin, M. Lagarde, E. Ruiz, A. Martinez, E. Bereciartua, F. Rodriguez, A. Arnaiz, M. Goneaga, N. Benito, J. Rodriguez-Baño, E. Espejo, M. Pujol. Predictive Factors for Overall Mortality (OM) in Patients with Methicillin-Resistant *Staphylococcus aureus* (MRSA). Bloodstream infection (BSI). Prospective Study in 22 Spanish Hospitals. 51th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC11). Chicago, 17-20 September 2011.

9. O.Gasch, M.Camoez, MA. Domínguez, B. Padilla, V.Pintado, B. Almirante, C.Martin, M. Lagarde E. Ruiz, JA. Martínez, E. Bereciartua, F. Rodríguez, A. Arnaíz, MA. Goenaga, N. Benito, J. Rodriguez-Baño, E. Espejo, M. Pujol on behalf of REIPI/GEIH study groups. Risk Factors and Outcomes of Infective Endocarditis (IE) among Methicillin resistant *Staphylococcus aureus* (MRSA) Bacteremia. Prospective Study in 21

Spanish Hospitals. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID12). London, 31 March-03 April 2012.

10. O. Gasch, M. Camoez, MA. Domínguez, B. Padilla, V. Pintado, B. Almirante, C. Martins, F. Lopez-Medrano, E. Ruiz, JR. Blanco, G. García-Pardo, E. Calbo, J. Horcajada, A. Granados, A. Jover, C. Dueñas and M. Pujol on behalf of REIPI/GEIH study groups. Daptomycin MIC increase among patients with Methicillin-Resistant *Staphylococcus aureus* (MRSA) Persistent Bacteremia (PB) Treated with Daptomycin. Prospective Study in 22 Spanish Hospitals. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID12). London, 31 March-03 April 2012.

11. M. Camoez, JM. Sierra, T. Conceição, M. Miragaia, H. de Lencastre, R. Martin, MA. Domínguez. Staphylococcal Chromosome Cassette mec (SCCmec) Stability in Major Endemic Methicillin-Resistant *Staphylococcus aureus* (MRSA) Clones Over 20 Years. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID12). London, 31 March-03 April 2012.

12. JM. Sierra, M. Camoez, I. Roca, J. Vila and MA. Dominguez. Use of MALDI-TOF to characterize different Clonal Complexes of methicillin-resistant *Staphylococcus aureus* (MRSA). Presentation number: D-1775. 52th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC12). San Franscico, 09-12 September 2012.

13. M. Camoez, JM. Sierra, F. Tubau, O. Gasch, M. Pujol, J. Liñares, R. Martin and MA. Domínguez. Accessory Gene Regulator (agr) Function, rpoB Mutations and Vancomycin (VA) Susceptibility in Methicillin-Resistant *Staphylococcus aureu*s (MRSA) Blood Isolates. 52th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC12). San Franscico, 09-12 September 2012

14. M. Camoez, O. Gasch, M. Pujol, JM. Sierra, E. Cercenado, E. Loza, N. Larrosa, E. Ruiz de Gopegui, F. Chaves, L. Martínez-Martínez, MA.Domínguez, on behalf of REIPI/GEIH study groups. Distribution of spa types among Methicillin-Resistant *Staphylococcus aureus* (MRSA)

bloodstream isolates collected between 2008 and 2009 in Spain. 23rd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID13). Berlin, 27-30 April 2013.

15. O. Gasch, S. Miyakis, M. Camoez, MA. Domínguez, M. Carrera, M. Plane, J. Dakos, GM. Eliopoulos, RC. Moellering. Development of mprF gene mutations in methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia with emerging resistance during treatment with daptomycin. 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC13). Denver, 10-13 September 2013.

16. M. Camoez, J. Càmara, JM. Sierra, F. Tubau , M. Pujol, MA. Domínguez. Methicillin-Resistant *Staphylococcus aureus* (MRSA) Causing Bacteremia in a Tertiary Spanish Hospital: Antibiotic Susceptibility and Molecular Epidemiology. 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC13). Denver, 10-13 September 2013.

17. M. Fernández-Ruiz, R. San-Juana, JM. Aguadoa, O. Gasch, M. Camoez, F. López-Medranoa, MA. Domínguez, B. Almirante, B. Padilla, M. Pujol on behalf of REIPI/GEIH Study Groups. Impact of elevated vancomycin minimum inhibitory concentrations on the outcome of catheter-related bloodstream infection due to methicillin-resistant *Staphylococcus aureus*. 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID14). Barcelona, 10-13 May 2014.

18. M. Camoez, J. Càmara, F. Tubau, M. Pujol, MA. Domínguez. Evolution of Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 isolates infecting or colonizing patients in a Spanish hospital: genotypes and clinical features. 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID14). Barcelona, 10-13 May 2014.

19. M. Camoez, J. Palacios, F. Tubau, M. Pujol, MA. Domínguez. Methicillin Resistant *Staphylococcus aureus* (MRSA) Clonal Replacement: Gradually Increase of CC8-MRSA in a Spanish Hospital. 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC14). Washington, 5-9 September 2014.

20. M. Camoez, F. Tubau, MA. Domínguez, S. Gomez-Zorrilla, C.Peña, C. Ardanuy. Molecular characterization of the two major clones of metallo-β-lactamases (MBL)-producing *Pseudomonas aeruginosa* (PA) in a Spanish hospital. 54th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC14). Washington, 5-9 September 2014.

B) Oral communications at international meetings

1. M. Camoez, JM.Sierra, M. Pujol, A. Hornero, R.Martin, MA. Domínguez. Prevalence and Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) ST398 over 12 years in a Spanish Hospital. Presentation number: O-270. 22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID12). London, 31 March-03 April 2012.

2. M. Camoez, MA. Domínguez, O. Gasch, E. Cercenado, N. Larrosa, F. Chaves, E. Loza, L. Martinez, M. Pujol on behalf of REIPI/GEIH(SEIMC). Methicillin-Resistant *Staphylococcus aureus* (MRSA) clones of community origin causing bacteremia in Spanish hospitals: a multicenter study. Presentation number: K-1982. 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC10). Boston, 12-15 September 2010.

C) National meetings

1. MA. Domínguez, V. Mick, M. Pujol, M. Camoez, J. Liñares, R. Martín. Sensibilidad antibiótica y caracterización genética de aislamientos de *Staphylococcus aureus* resistentes a meticilina (SARM) de origen comunitario. Poster nº215. XIII Reunión de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Sevilla, 3-5 June 2009.

2. O. Gasch, MA. Domínguez, M. Camoez, M. Lagarde, J. Rodríguez-Baño, B. Almirante, B. Padilla, V. Pintado, M. Pujol. Bacteriemia por *Staphylococcus aureus* resistente a la meticilina (SARM). Análisis

preliminar de 612 episodios. Estudio Multicéntrico REIPI/GEIH (SEIMC). Poster nº204. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Barcelona, 19-22 May 2010.

3. O. Gasch, MA. Domínguez, M. Camoez, M. Lagarde, J. Rodríguez-Baño, B. Almirante, B. Padilla, V. Pintado, M. Pujol. Monitorización de la concentración plasmática de vancomicina (CPV) en el tratamiento de la bacteriemia por *Staphylococcus aureus* resistente a meticilina (SARM). Análisis de 428 pacientes. Estudio Multicéntrico REIPI/GEIH (SEIMC). Poster nº203. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Barcelona, 19-22 May 2010.

4. O. Gasch, MA. Domínguez, M. Camoez, M. Lagarde, J. Rodríguez-Baño, B. Almirante, B. Padilla, V. Pintado, M. Pujol. Uso de Daptomicina en la Bacteriemia por *Staphylococcus aureus* resistente a meticilina (SARM). Estudio Multicéntrico REIPI/GEIH (SEIMC). Poster nº202. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Barcelona, 19-22 May 2010.

5. M. Camoez, MA. Domínguez, Z. Vásquez, M. Pujol, R. Martín. PCR a tiempo real para la detección de portadores de *S. aureus* resistente a meticilina. Aplicación a pacientes en descontaminación nasal con mupirocina. Poster nº764. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Barcelona, 19-22 May 2010.

6. MA. Domínguez, M. Camoez, O. Gasch, Z. Vásquez, E. Cercenado, N. Larrosa, F. Chaves, E. Loza, L. Martínez-Martínez, M. Pujol. *Staphylococcus aureus* resistente a meticilina (SARM) causante de bacteriemia: sensibilidad antibiótica y epidemiología molecular. XIV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Barcelona, 19-22 May 2010

7. JM Sierra, M Camoez, M Pujol, R Martin, MA Dominguez. Análisis de los genes de virulencia en clones de *Staphylococcus aureus* resistente a meticilina causantes de bacteriemia. XV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Málaga, 1-4 June 2011.

8. M. Camoez, MA. Domínguez, A. Fernandez JM. Sierra, J. Ayats, F. Tubau, O. Gasch, M. Pujol, R. Martin, J. Liñares. Actividad bactericida in vitro de vancomicina y daptomicina frente a cepas de diferentes clones de *Staphylococcus aureus* resistente a meticilina causantes de bacteriemia. Hospital de Bellvitge (Barcelona, 1990-2009). Poster nº488. XV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Málaga, 1-4 June 2011.

9. M. Camoez, JM. Sierra, O. Gasch, M. Pujol, E. Cercenado, E. Loza, N. Larrosa, E. Ruiz de Gopegui, F. Chaves, L. Martínez-Martínez, MA.Domínguez, Por el grupo de trabajo de REIPI y GEIH. Sensibilidad a linezolid en cepas de *Staphylococcus aureus* resistentes a meticilina aisladas en pacientes con bacteriemia. Estudio Multicéntrico REIPI/GEIH (SEIMC). Poster nº487. XV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Málaga, 1-4 June 2011.

10. M. Camoez, JM. Sierra, F. Tubau, O. Gasch, M. Pujol, J. Liñares, R. Martin and MA. Domínguez. Estudio del polimorfismo del operón agr y sensibilidad a vancomicina (VA) en cepas de *Staphylococcus aureus* resistente a meticilina (SARM) causantes de bacteriemia en el Hospital Universitari de Bellvitge (HUB). Poster nº584. XVI Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Bilbao, 9-11 May 2012.

11. M. Camoez, MJ. Peña, M. Pujol, R. Fernandez, A. Hornero, R. Martin, MA. Domínguez. Evaluación de un sistema comercial de PCR a tiempo real para la detección temprana de portadores nasales de *S. aureus* resistente a meticilina. Presentation number: 570. XVII Congreso de la

Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Zaragoza, 29-31 May 2013.

12. A Imaz, S Di Yacovo, M Camoez, O Gasch, MA Dominguez, M Maso-Serra, A Vila, M Pujol, D Podzamczer. Estudio de la prevalencia de colonización por *Staphylococcus aureus* resistente a meticilina de adquisición comunitaria en una cohorte de pacientes con infección por VIH. XVII Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Zaragoza, 29-31 May 2013.

Annex III

Questionaire

A.- Identificación y datos paciente/cepa

Nombre Centro:

Código Centro (CC) (2 cifras): (_ ,_) Iniciales Nombre, 1º Apellido (N,A):(_ ,_)

Sexo (S): (0) Hombre; (1) Mujer Fecha Bacteriemia (FB) (dd/mm/aa):

Código IDENTIFICACION CEPA: (C,C.N,A,S,FB) (_,_,_,_,_,_,_,_,_,_)

B.- Sensibilidad antibiótica

B.1.- Método de identificación de la resistencia a meticilina:

Antibiograma (__) Látex PBP 2A (__) PCR (__): Comercial (_)
Casera (_)

Otros métodos: _____

B.2.- Sensibilidad antibiótica

Antimicrobiano	mm (disco difusión)	S ó R	μg/mL (*)	S ó R
Penicilina				
Oxacilina				
Cefoxitina				
Eritromicina				
Clindamicina				
Cotrimoxazol				
Cloramfenicol				
Gentamicina				
Tobramicina				
Amikacina				
Tetraciclina				
Rifampicina				
Ciprofloxacina				
Levofloxacina				
Vancomicina				
Teicoplanina				
Mupirocina				
Quinupristina/ Dalfopristina				
Linezolid				
Daptomicina				

(*) Método: Microdilución (__) E-test (__) Otros:_____

B.3.- Se realiza algún método de **cribaje de sensibilidad disminuida a glicopéptidos:**

No (__) Sí (__): Cual _____

B.4.- OBSERVACIONES