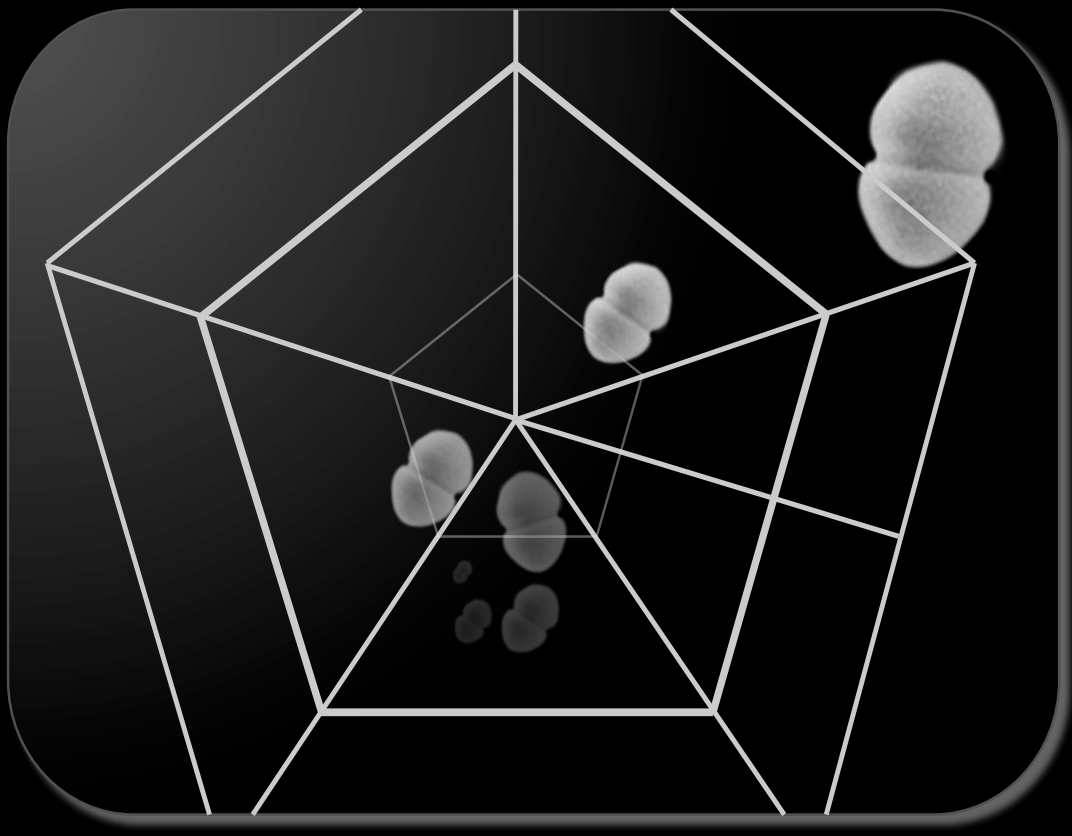
Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Portugal:

Origin, epidemiology and virulence

Ana Lopes Tavares



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
December 2014

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
/UNL

Knowledge Creation

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Portugal:

Origin, epidemiology and virulence

Ana Lopes Tavares

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras, December, 2014

INSTITUTO
DE TECNOLOGIA
QUÍMICA E BIOLÓGICA
/UNL

Knowledge Creation

Second edition, January 2015

ISBN: 978-989-20-5135-2

Supervisors:

Dr. Hermínia de Lencastre

Dr. Maria Miragaia

Chairman of Examiners:

Dr. Cecília Arraiano, Full Professor at ITQB

Examiners:

Dr. Constança Pomba

Dr. João Paulo Gomes

Dr. José Melo Cristino

Dr. Isabel Couto

Dissertation presented to obtain the PhD degree in Biology by Universidade Nova de Lisboa, Instituto de Tecnologia Química e Biológica.

ACKNOWLEDGEMENTS

To Dr. Hermínia de Lencastre, Professor and Head of the Laboratory of Molecular Genetics (Microbiology of Human Pathogens Unit) at Instituto de Tecnologia Química e Biológica António Xavier (ITQB), Universidade Nova de Lisboa (UNL), my supervisor, for accepting me in her Laboratory and welcoming me into her group. For her vast scientific knowledge and fascinating carrier in the field of staphylococci; she is a worldwide reference concerning the molecular epidemiology and resistance mechanisms of *Staphylcoccus aureus*.

To Dr. Maria Miragaia, Auxiliary Investigator and Head of Laboratory of Bacterial Evolution and Molecular Epidemiology (Microbiology of Human Pathogens Unit) at ITQB/UNL, my co-supervisor, for guiding me trough the first steps in the molecular characterization of the staphylococci, for teaching me how to be critical in science, for her incentive during the not so good moments, and for her patience and valuable critical points of view throughout this work and along the years. Thanks for her friendship.

To Dr. Henrik Westh, Associate Professor, Senior Consultant Microbiologist, in the Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen University Hospital, my co-supervisor for his generosity and for welcoming me at the Department of Clinical Microbiology, where part of the work included in this Thesis, regarding alpha-hemolysin studies, was performed. Thanks for providing me all the resources that I needed for the development of the work, and also for making me feel at home.

To Dr. Isabel Couto, Assistant Professor at Instituto de Higiene e Medicina Tropical (IHMT) at UNL, who guide me through the first steps of the unbelievable world of *S. aureus*. For the person she is, her scientific knowledge and for the unconditional dedication and recognition of her students. Thanks for the friendship and confidence deposited in me, for the interesting discussions we had and for the encouraging words and help throughout my master Thesis.

To Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa for providing excellent conditions for the development of my PhD studies.

To Fundação para a Ciência e Tecnologia (FCT) (SFRH/BD/44220/2008), European Union (CONCORD-HEALTH-F3-2008/ Project Number 222718/ European Commision) and to Fundação Calouste Gulbenkian (037/BI-BI/2012) for the financial support during this PhD.

To all my colleagues and friends at the LGM along all these years, for their friendship and for sharing with me the passion for science and, in some cases, the enthusiasm for such peculiar "bug" as *Staph aureus*: Alexandra Simões, Ana Gomes, Bruno Guerra, Céline Coelho, Diana Espadinha, Carina Valente, Débora Tavares, Inês Crisóstomo, Inês Grilo, Liliana Curto, Juliana Lamaro, Maria Luís Amorim, Nuno Faria, Ons Bouchami, Pedro Arede, Raquel Portela, Rita Sobral, Sonia Nunes, Sónia Almeida, Sofia Felix, Susana Gardete, Teresa Conceição, Teresa Figueiredo and Indiara Sales.

To my friends Joana Rolo, Catarina Milheiriço and Nelson Frazão for sharing with me so many good moments, for the enthusiastic discussions we had and, above all, for their friendship.

To Ana Cristina Paulo, very special thanks, not only for her precious help in finding consistency in the alpha-hemolysin data, but also for her friendship and persistence in helping me during the very difficult personal moment she was living.

To Cândida Delgado and Helen Accuri for their prompt and unconditional help in the Herculean task of searching and rescuing data from the S3DB.

To "Dona Isilda", for being as a "mother" to all of us, always available to help along all these years. For sharing with me the happiness and sadness of life, and for her kindness and friendship.

To "Dona Manuela", for always taking care of the hardest part of our work (bureaucracy), for her help and precious advices through all these years.

To Professor Ana Madalena, although we had not worked together, I thank her for being always kind and pleasant to me.

To Dr. Marta Aires de Sousa and Dr. Raquel Sá-Leão for the knowledge they shared with me in my very early times at Laboratory of Molecular Genetics, and also for their kindness. Special thanks to Raquel Sá-Leão for her continuous attention to my carrier and personal life.

To Sissel Skovgaard, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen University, the "special" person I was very lucky to meet in Copenhagen. For receiving me and providing all the help I needed in the development of the part of the work, regarding growth curves, performed at Life University. Thanks for making this part of the work possible; she was always there for helping me, with her kindness and smile. Thanks for hosting me, but mostly thanks for the long life friendship.

To Kristian Schønning and Jesper Nielsen, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen University, for their enthusiastic and productive discussions about part of Thesis studies related with the alpha-hemolysin (*hla*) expression. For their friendship and persistent support. Special thanks to Jesper, a precious help in the bench work, who was always there when I needed.

To Kit Boye and Susanne Rohde, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen University, for their friendship and kindness. For their help in particular in the *hla* sequencing. I would have never finished the work without them.

To Mette Hogh, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen University, for her "remote" help in the optimization of RNA extraction and probe design.

To my "old" friends Lidia, Daniela and Sofia for sharing so many good moments, while growing together with me.

To my new (kindergarten) friends, Madalena, João, Dudu and Sofia and Mane, Woytek, Helena and Bernardo. Having children has many benefits, and meet you was one special "benefit" that Adriana gave to me. Thanks for the past, present and future good moments.

To my “new family” Ivone, Helder, Susana, Rui, Beatriz and Madalena for the very good moments we have had and for always being ready to help.

To my small and special family for their unconditional support and love, my parents Terezinha and Zé, to my sister Cristina and brother in law Ricardo and my nephews Francisco, Gaspar e Emilia. To all of them, my apologies for having declined some of our family meetings because of the work of the Thesis. “The gift is ready”.

And finally, to Ricardo and Adriana, the joys of my life and to whom this thesis is dedicated. Thank you for existing and making my life so special.

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA), a human pathogen confined to hospitals (HA-MRSA) for over 30 years have been emerging worldwide in the last two decades as a leading cause of severe infections in healthy individuals in the community (CA-MRSA). Despite its clinical significance, in the beginning of our studies no information existed on the prevalence, and population structure of CA-MRSA in Portugal. Moreover, it remained to be clarified how CA-MRSA emerged in our country. In particular, it was not known if CA-MRSA emerged locally by acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) by established methicillin-susceptible *S. aureus* (MSSA) in the community, if they were imported from abroad or have escaped from the hospital.

CA-MRSA have specific genetic backgrounds, different from their hospital counterparts, and its success as a pathogen in the community has been related with an enhanced virulence potential believed to be associated to the presence and differential expression of specific virulence factors like Panton-Valentine leukocidin (PVL), alpha-hemolysin (Hla), arginine catabolic mobile element (ACME) and phenol soluble modulins (PSMs). Notwithstanding, no extensive study was ever performed where expression of virulence genes was compared between CA-MRSA and HA-MRSA genetic backgrounds.

In this Thesis we described for the first time the prevalence and population structure of CA-MRSA in Portugal, provided clues on the origin of CA-MRSA in our country and produced new data that contribute to a better understanding of CA-MRSA pathogenic potential.

To understand the clinical relevance and origin of CA-MRSA in Portugal, a large and representative collection of both MSSA and MRSA with community origin was screened for the presence of the beta-lactam resistance determinant (*mecA*) and the virulence genes PVL, and ACME. Moreover, *S. aureus* genetic backgrounds were determined by a combination of state-of-the-art typing techniques, including SCC*mec* typing, pulsed-field gel electrophoresis (PFGE), *spa* typing and multilocus sequence typing (MLST).

The results obtained showed a high frequency of MRSA in the community (21.6%). However, only a small proportion was represented by typical CA-MRSA clones (11.4%). Surprisingly, the vast majority of the MRSA in the community were HA-MRSA clones (88.6%).
A high genetic diversity among the CA-MRSA was identified, represented by more epidemic (USA300, USA400, USA700, Southwest Pacific (SWP), European, and ST398) and less disseminated (ST1810) clones. In contrast, among the HA-MRSA mostly only two epidemic clones were found, the EMRSA-15 clone (77.2%) and the New York/Japan (NY/JP) (14.9%) – coincidently, the two most prevalent clones in Portuguese hospitals. Altogether, these findings indicate that the high prevalence of MRSA in the community in Portugal seem to result mainly from dissemination of HA-MRSA clones from hospitals.

As expected, the MSSA population in the community was more prevalent (78.4%) and a higher genetic diversity was observed when compared to the MRSA. Likewise, we found MSSA related to both CA-MRSA (50.8%) and HA-MRSA (49.1%) genetic backgrounds. A considerable proportion of MSSA (46.7%) were related with the CA-MRSA epidemic clones USA700 (30.3%), USA400 (26.3%), USA300 (18.2%), ST398 (14.1%), SWP (8.1%) and Taiwan (3%). Concerning the MSSA related with HA-MRSA epidemic clones, the great majority (92.6%) were related to EMRSA-16 (38.4%), NY/JP (27.6%), Berlin (15.1%), EMRSA-15 (9.7%), and the Pediatric clone (9.2%). These results suggest that in the community the MSSA population is highly related to the MRSA population, and that in this environment SCC*mec* can be frequently acquired by MSSA and/or lost by MRSA.

To further understand how the emergence of MRSA might have shaped the MSSA population in Portugal, we have analyzed the MSSA population dynamics and geographical distribution over almost two decades in the community and hospital in Portugal. The molecular characterization of MSSA isolates, with community and hospital origin, collected over a 19 year-period (1992–2011) by state-of-the-art molecular typing techniques showed that MSSA were genetically diverse. However, one major clone (ST30-t012) was present in the entire study period and all over the country, and other clones (ST5-t002, ST8-t008, ST15-t084, ST34-t166, ST72-t148, ST1-t127, ST7-t091 and ST398-t571) were intermittently detected over time. Moreover, we found that MSSA isolates with genetic backgrounds related to CA-MRSA clones (ST8-t008, ST72-t148 and ST1-t127) appeared after the report of the first cases CA-MRSA infections in 1989 only. These results confirm that the

changes occurring in MRSA epidemiology might have impact on MSSA epidemiology.

To evaluate the variation in virulence gene expression in CA and HA genetic backgrounds, we analyzed one of the most common and clinically relevant virulence factors in *S. aureus*, the alpha-hemolysin (Hla). The nucleotide sequences of the *hla* gene and its promotor and the relative expression in a large and representative collection of epidemic and minor MRSA/MSSA of both community and hospital origin were analyzed by DNA sequencing and Real-Time PCR (RT-PCR), respectively. The results indicated that the *hla* gene has evolved together with the genetic background, but the same could not be observed for the *hla* promotor region. Although no correlations could be established between allotypes and expression profiles, the data obtained suggest that CA backgrounds demonstrated, in general, a higher *hla* expression than HA backgrounds. Notwithstanding, a high isolate-to-isolate variation in the level of gene expression was detected in highly related isolates. Moreover, a high *hla* expression was observed in two isolates belonging to the EMRSA-15 and NY/JP clones, both nosocomial MRSA clones found to be predominant in Portuguese hospitals. These observations highlighted the need of including a diverse and representative isolate collection, while evaluating the CA-MRSA pathogenesis, in future studies. Moreover, we found that the two most predominant HA-MRSA clones in the hospitals and community in Portugal have a high pathogenic potential.

RESUMO

Os *Staphylococcus aureus* resistentes à meticilina (MRSA), um agente patogénico humano confinado aos hospitais (HA-MRSA) durante mais de 30 anos, emergiu nas últimas duas décadas em todo o mundo como uma das principais causas de infecções graves em indivíduos saudáveis na comunidade (CA-MRSA). Apesar da sua importância clínica, no início dos nossos estudos a informação sobre a prevalência e estrutura populacional dos CA-MRSA em Portugal era quase inexistente. Adicionalmente, não se conhecia a forma como os CA-MRSA emergiram no nosso país. Em particular, desconhecia-se se os CA-MRSA surgiram localmente pela aquisição do elemento genético móvel SCC*mec* (de *staphylococcal chromosome cassette mec*) que transporta o gene *mecA*, por isolados *S. aureus* susceptíveis à meticilina (MSSA), ou se, alternativamente, foram importados do exterior ou tiveram origem em estirpes disseminados do hospital.

Os isolados de CA-MRSA pertencem a tipos clonais específicos, que são diferentes daqueles encontrados no ambiente hospitalar, e o seu sucesso como agentes patogénicos na comunidade tem sido atribuído ao facto destas estirpes terem um maior potencial patogénico. Foi demonstrado que a virulência dos CA-MRSA está associada à presença e à expressão diferencial de determinados factores de virulência, nomeadamente a leucocidina Panton-Valentine (PVL), a alfa-hemolisina (HLA), o elemento ACME (de *arginine catabolic mobile element*) e as PSMs (de *phenol soluble modulins*). Não obstante, ainda não foi realizado nenhum estudo alargado, onde se tivesse comparado a expressão de genes de virulência entre os diferentes tipos clonais de CA-MRSA e HA-MRSA.

Neste estudo, descrevemos pela primeira vez a prevalência e a estrutura da população dos CA-MRSA em Portugal, sugerimos as possíveis origens dos CA-MRSA no nosso país e obtivemos novos dados que contribuem para uma melhor compreensão do potencial patogénico dos CA-MRSA.

Para compreender a relevância clínica e a origem dos CA-MRSA em Portugal, foi estudada a presença do determinante de resistência aos beta-lactâmicos (*mecA*) e dos genes de virulência PVL e ACME numa colecção ampla e representativa de isolados MSSA e MRSA com origem na comunidade. Determinaram-se os tipos clonais dos isolados de *S. aureus* utilizando diversas técnicas de tipagem, nomeadamente a determinação da estrutura do SCC*mec*, a electroforese em

campo pulsado (PFGE, *de pulsed-field gel electrophoresis*), a sequenciação nucleotídica de um fragmento interno do gene *spa* e de sete genes nativos (MLST, de *multilocus sequence typing*).

Os resultados obtidos mostraram uma elevada frequência de MRSA na comunidade (21,6%). No entanto, verificámos que apenas uma pequena proporção dizia respeito a tipos clonais característicos da comunidade ou CA-MRSA (11,4%). A vasta maioria dos isolados de MRSA existentes na comunidade em Portugal pertenciam a tipos clonais característicos do hospital ou HA-MRSA (88,6%).

Identificámos uma elevada diversidade genética nos isolados pertencentes a tipos clonais associados à comunidade (CA-MRSA), incluindo clones considerados epidémicos (USA300, USA400, USA700, Sudoeste do Pacífico (SWP), Europeu e ST398) e clones menos epidémicos (ST1810). Em contraste, apenas dois clones epidémicos foram encontrados entre os MRSA pertencentes a tipos clonais associados ao hospitais (HA-MRSA), em especial o tipo clonal EMRSA-15 (77,2%), mas também o tipo clonal Nova Iorque/Japão (NY/JP) (14,9%) - coincidentemente, os dois clones com maior prevalência nos hospitais portugueses.

Em resumo, estes resultados indicam que a elevada prevalência de MRSA na comunidade em Portugal parece resultar, principalmente, da disseminação de isolados pertencente a tipos clonais tipicamente hospitalares.

Apesar da elevada frequência de MRSA na comunidade, a grande maioria da população de *S. aureus* encontrada neste ambiente era susceptível aos antibióticos beta-lactâmicos (MSSA) (78,4%), apresentando uma maior diversidade genética. Tal como foi descrito para os isolados de MRSA, encontrámos MSSA relacionados com tipos clonais característicos de CA-MRSA (50,8%) mas também isolados relacionados com tipos clonais característicos de HA-MRSA (49,1%). Uma considerável proporção de MSSA (46,7%) estavam relacionados com os tipos clonais epidémicos de CA-MRSA, USA700 (30,3%), USA400 (26,3%), USA300 (18,2%), ST398 (14,1%), Southwest Pacific (SWP) (8,1%) e Taiwan (3%). Quanto aos MSSA relacionados com tipos clonais epidémicos dos hospitais (HA-MRSA), a grande maioria (92,6%) era semelhante ao tipos clonais EMRSA-16 (38,4%), NY/JP (27,6%), Berlim (15,1%), EMRSA-15 (9,7%) e o clone Pediátrico (9,2%). Estes resultados sugerem que na comunidade a população de MSSA está muito relacionada com a população de MRSA, e que neste ambiente o SCC*mec* pode ser frequentemente adquirido pelos isolados de MSSA e/ou perdidos pelos MRSA.

Para compreendermos o impacto que a emergência dos isolados de MRSA na comunidade em Portugal poderá ter tido na estrutura da população de MSSA, analisámos a dinâmica populacional dos MSSA e a distribuição geográfica ao longo de quase duas décadas em Portugal, na comunidade e no hospital.

A caracterização molecular dos isolados de MSSA, com origem na comunidade e hospitalar, recolhidos ao longo de um período de 19 anos (1992-2011), pelas técnicas de tipagem actualmente usadas para *S. aureus*, mostraram que os MSSA são genéticamente diversos. No entanto, verificou-se haver um clone maioritário (ST30-t012) que esteve presente ao longo de todo o período de estudo e em todo o país, em contraste com outros clones (ST5-t002, ST8-t008, ST15-t084, ST34-t166, ST72-t148, ST1-t127, ST7-t091 e ST398-t571) que foram detectados de forma intermitente ao longo do tempo. Adicionalmente, verificámos que os isolados de MSSA genéticamente relacionadas com tipos clonais característicos da comunidade (ST8-t008, ST72-t148 e ST1-t127) apareceram somente depois das primeiras descrições de CA-MRSA no mundo (1989). Esses resultados sugerem que as mudanças que ocorrem na epidemiologia de MRSA poderão ter impacto sobre a epidemiologia de MSSA.

Para avaliar a variação na expressão dos genes de virulência em tipos clonais característicos da comunidade e hospital, analisámos a alfa-hemolisina (Hla), um dos factores de virulência mais comuns e clinicamente mais relevantes em *S. aureus.* As sequências nucleotídicas do gene *hla* e do seu promotor assim como a expressão relativa do gene foi determinada por sequênciação do DNA e por PCR em tempo real (RT-PCR), respectivamente, numa colecção vasta e representativa de MRSA/MSSA epidémicos e não epidémicos, com origem na comunidade e no hospital.

Os nossos resultados mostraram que o gene *hla* evoluiu em paralelo com as linhagens genéticas, no entanto, o mesmo não se observou para a região do promotor do *hla*. Apesar de não se poder estabelecer qualquer tipo de correlação entre os diferentes alelos do gene *hla* e do seu promotor com os perfis de expressão, os dados obtidos sugerem que os isolados pertencentes a tipos clonais epidémicos típicos da comunidade demonstraram, em geral, uma maior expressão de *hla* do que os tipos clonais mais frequentemente encontrados nos hospitais. Não obstante, observou-se uma grande variação no nível de expressão do gene em isolados geneticamente muito relacionados. Adicionalmente, observou-se que dois isolados pertencentes aos clones EMRSA-15 e NY/JP, ambos tipos clonais frequentemente encontrados nos hospitais portugueses, tinham uma expressão relativamente elevada.

Estes resultados sugerem que estudos futuros focados na avaliação do potencial patogénico de MRSA não deverão cingir-se à análise de apenas um isolado, mas deverão incluir a análise de uma colecção de isolados diversificada e representativa. Adicionalmente, os nossos dados mostraram que os dois clones de HA-MRSA mais predominantes nos hospitais e na comunidade em Portugal têm também um elevado potencial patogénico, o que é particularmente preocupante.

THESIS OUTLINE

In **Chapter I**, ***General Introduction*****,** a general overview is provided on the current knowledge of the *S. aureus* epidemiology, with particular emphasis on community-associated *Staphylococcus aureus* (CA-MRSA); the genetic basis of methicillin resistance and virulence in *S. aureus* are also introduced.

Chapters II, III and IV include results from three different studies that analyzed the prevalence, population structure and origin of CA-MRSA in Portugal, the relatedness of methicillin-resistant and methicillin-susceptible *S. aureus* (MRSA and MSSA) populations, the dynamics of MSSA population overtime and the variation in gene expression of one of the most important *S. aureus* virulence factor (alpha-hemolysin).

In **Chapter II**, the study *"High prevalence of hospital-associated methicillin-resistant Staphylococcus aureus in the community in Portugal: evidence for the blurring of community–hospital boundaries"*, was focused on the molecular epidemiology and relatedness of MSSA/MRSA in the community in Portugal. *S. aureus* isolates and epidemiological data were collected from representative hospitals in Portugal and analyzed by state-of-the-art typing techniques. We described the prevalence and population structure of MSSA and MRSA isolates causing infections in the healthy individuals in the community in Portugal. This study reveals a considerable frequency of MRSA in the Portuguese community, although the majority of population was composed by MSSA. The data obtained showed that the main MRSA clones circulating in the community have a hospital origin (HA-MRSA), and only a small proportion was represented by CA-MRSA clones. Moreover, almost all epidemic MRSA present in the community were related with contemporary MSSA.

In **Chapter III**, the study "*Population structure of methicillin-susceptible Staphylococcus aureus (MSSA) in Portugal over a 19 years period (1992 to 2011)"* includes the analysis of MSSA population structure overtime in Portugal. MSSA isolates collected over a 19 year-period in Portugal were analyzed by state-of-the-art techniques. We defined variations of MSSA population occurring over time in Portugal. Moreover, we inferred possible relatedness between MSSA and MRSA. This study evidenced that despite genetically diverse, some dominant MSSA clonal types have been established and widely disseminated for almost two decades. The study also allowed identifying

MSSA genetic backgrounds that could have been recipients of SCC*mec*, giving rise to local emergence of CA-MRSA clones in Portugal.

In **Chapter IV**, the study "*Insights into the evolution and gene expression of alpha-hemolysin (hla) among Staphylococcus aureus with hospital and community origin"*, aimed to compare the genetic diversity and gene expression of alpha-hemolysin, one of the most important virulence factors in *S. aureus*, in different genetic backgrounds of CA-MRSA/MSSA and HA-MRSA/MSSA. A collection of well-characterized and representative MRSA isolates with community and hospital origins with diverse geographical distribution and clinical origin was gathered. Isolates were analyzed for nucleotide diversity of *hla* gene and promoter gene by sequencing and for gene expression by RT-PCR. This study demonstrated that the *hla* genetic diversity is evolving with the species, although the promoter gene showed some variation to this pattern. Moreover, we confirmed that, in general, isolates belonging to CA clones showed higher levels of *hla* expression than isolates belonging to HA clones. However, we observed high isolate-to-isolate variation in the level of gene expression, which was independent of the genetic background.

In **Chapter V**, ***Discussion*** the major findings of this Thesis are highlighted and discussed. Integrating all the molecular epidemiological data, we propose a model for the origin of MRSA in the community. Moreover, we suggest a new paradigm where virulence features should be considered to be isolate-specific and not extrapolated for a group of strains with related genetic backgrounds.

ABBREVIATIONS

A

ACME – arginine catabolic mobile element

ADAM10 – disintegrin and metalloprotease domain-containing protein 10

Agr – accessory gene regulator

AFLP – amplified fragment length polymorphism

arcC - carbamate kinase

aroE - shikimate dehydrogenase

B

BURP – based upon repeat pattern

BURST – based upon related sequence types

C

CA – community-associated

CC – clonal complex

ccr – cassette chromosome recombinase

CDC – Centers for Disease Control and Prevention

Clf – clumping factors

Coa – collagen-binding protein

CV – core variable

D

D-Ala-D-Ala – D-alanyl-D-alanine

DDD – defined daily dose

DGS – Direcção Geral de Saúde

DLV – double locus variant

DNA – deoxyribonucleic acid

E

EARS-Net – European Antimicrobial Resistance Surveillance Network

EARSS – European Antimicrobial Resistance Surveillance System

EbpS – elastin-binding protein S

ECDC – European Centre for Disease Prevention and Control

EMRSA – epidemic methicillin-resistant *Staphylococcus aureus*

ESAC-Net - European Surveillance of Antimicrobial Consumption Network

F

FnBP – fibronectin-binding protein

G

G – guanidine

Gb – giga base pairs

GEN – gentamicin

goeBURST – global optimal eBURST

glpF - glycerol kinase

gmK - guanylate kinase

H

h – heterogeneous

HA– hospital-associated

HCW – healthcare worker

HIV - human immunodeficiency virus

Hla – alpha-hemolysin

hVISA – heterogeneous vancomycin-intermediate *S. aureus*

I

ITQB – Instituto de Tecnologia Química e Biológica

IS – insertion sequence

IWG-SCC – International Working Group on the Staphylococcal Cassette Chromosome Elements

J

J – joining/junkyard regions

JP – Japan

M

m – messenger

MDR – multidrug resistant profile

MGE – mobile genetic element

MIC - minimum inhibitory concentration

MLS – macrolide, lincosamide and streptogramin

MLST – multilocus sequence typing

MLVA - multiple locus variable-number tandem repeat analysis

MSSA - methicillin-susceptible *Staphylococcus aureus*

MRSA – methicillin-resistant *Staphylococcus aureus*

MSCRAMM – microbial surface components recognizing adhesive matrix molecules

N

NGS – next generation sequencing

NICU - neonatal intensive care units

NY – New York

O

OXA – oxacillin

P

p – plasmid

PBP – penicillin-binding protein

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PSM – phenol soluble modulin

PVL – Panton-Valentine leukocidin

***pta* -** phosphatase acetyltransferase

R

RAPD - Random Amplified Polymorphic DNA

RIF – rifampicin

RNA – ribonucleic acid

RT-PCR – real-time reverse transcriptase PCR

S

SaeRS– *S. aureus* exoprotein regulator

SAg –superantigen

SarA – staphylococcal accessory regulator

SaPIs – pathogenicity islands

SCC– staphylococcal cassette chromosome

SCC*mec* – staphylococcal cassette chromosome *mec*

SCV – small colony variants

SEs – staphylococcal enterotoxins

SLV – single locus variant

SNPs – single nucleotide polymorphisms

spa – *Staphylococcus aureus* protein A

SSSS – staphylococcal scalded skin syndrome

SSTIs – skin and soft tissue infections

ST – sequence type

SWP – Southwest pacific

T

TCSs – two-component systems

Tn – transposon

TSS – toxic shock syndrome

tpi - triosesphonate isomerase

U

UK – United Kingdom

USA – United States of America

V

VISA – vancomycin-intermediate *S. aureus*

VRSA – vancomycin-resistant *S. aureus*

W

WGS – whole-genome sequencing

Y

***yqiL*-** acetyl coenzyme A acetyltransferase

TABLE OF CONTENTS

CHAPTER IV

CHAPTER V

CHAPTER I

General Introduction

CHAPTER I

General Introduction

1. *Staphylococcus aureus*

GENERAL FEATURES

Staphyloccoccus aureus was first discovered in 1880 in a human abscess pus by the surgeon Sir Alexander Ogston, who after the observation of its characteristic grape-like clusters, named them *Staphylococcus* from the Greek expression "staphylé" (a bunch of grapes) (267). Four years later (1884), Rosenbach was able to isolate and grow these bacteria also from abscesses and called them *Staphyloccocus aureus*, because of the gold-like pigmentation appearance of the colonies ("*aureus*" from Latin "golden") (304) in opposition to *S. albus* ("albus" from Latin "white), nowadays known as *S. epidermidis*.

S. aureus belongs to a distinct monophyletic group within the *Firmicutes* Phylum, to the class *Bacilli*, order *Bacillales,* family *Staphylococcaceae and* genus *Staphylococcus* (123, 281, 340). Presently, the genus *Staphylococcus* contains 49 species and 26 subspecies (http://www.bacterio.net/s/staphylococcus.html accessed on September 2014). *S. aureus* are a Gram-positive cocci, with 0.5 to 1.5 µm diameter, that can contain or not a polysaccharide capsule, are non-motile and non-sporeforming, facultative anaerobes, which produce catalase and coagulase (266, 393).

GENOME

The *S. aureus* genome consists of a circular chromosome of approximately 2,700 to 2,900 Mb, with low G+C content (32.8%) (17). There are currently 56 annotated complete whole-genome sequences of *S. aureus* available in the public domain (http://www.ncbi.nlm.nih.gov/genome/genomes/154? assessed on September 2014). The genome is composed of the core genome and the accessory genome (205).

The core genome encodes genes essential for bacterium cell growth and survival, and includes housekeeping genes, genes associated with metabolism, but also virulence genes, as genes coding for the capsule, surface associated proteins and toxins. Variations in the sequence of core genome is observed – core variable (CV) – and may result from single nucleotide polymorphisms (SNPs) to

larger regions of DNA diversity (from few nucleotides within a gene to insertion/deletion of several kilobase pairs) (205). Core genes are located on the bacterial chromosome and, therefore, are typically stable and transferred vertically.

The accessory genome represents up to 25% of the *S. aureus* genome and includes bacteriophages, plasmids, *S. aureus* pathogenicity islands (SaPI), transposons and staphylococcal cassette chromosome (SCC) elements (107, 204, 205). The accessory genome largely contributes to the high genetic and phenotypic plasticity of *S. aureus* and mainly contains resistance and virulence genes. The accessory genes can be transmitted by horizontal transfer to other strains and species, but also vertically to the daughter cells (205).

OPPORTUNISTIC PATHOGEN IN THE HOSPITAL AND COMMUNITY

S. aureus is a remarkable versatile bacterium with a two-faced lifestyle. On one hand, *S. aureus* behaves as a harmless colonizer, on the other hand is one of the most successful human pathogens and a worldwide leading cause of infections.

S. aureus can exist as a commensal (251) and colonizer of skin and mucous membranes of humans and animals, including, pigs, cattle, rabbits, dogs and cats among others (212, 251). Despite being found in multiple human body sites, colonization of the anterior nares is the preferential ecological niche (79, 367, 385). Notwithstanding, some studies indicated that the rate of throat colonization is higher than colonization of the anterior nares (221, 258).

Studies addressing nasal colonization in healthy individuals showed that about 20% of healthy individuals are persistent nasal carriers, while 30% are intermittently colonized, and 50% are non-carriers (79, 385). The prevalence of *S. aureus* nasal carriage showed to vary in different populations in the community. The colonization rate in children has been reported to be significantly higher when compared with adults (385). Moreover, carriage rates were found to vary between countries. A recent structured survey studying *S aureus* nasal carriage in healthy individuals aged 4 years or older in nine European countries (Austria, Belgium, Croatia, France, Hungary, Spain, Sweden, the Netherlands and the UK) showed that great variations in carriage rates in healthy patients exist between countries, varying from 12.1% in Hungary to 29.4% in Sweden (80). In Portugal, the rate of *S. aureus* nasopharyngeal carriage was recently reported to be 17.4% among

children attending day-care centers (344). Moreover, the authors observed that the carriage rate was associated with age, ranging from 6.3% in children less than two years old to up to 27.5% among six years old children (344).

Frequent exposure to health care facilities and/or hospitalization are commonly associated with an increased rate of *S. aureus* colonization, most of the time with *S. aureus* carrying antimicrobial resistance determinants. Among hospitalized individuals in the USA, prevalence of *S. aureus* nasal colonization was 28.6% in 2003-2004 (132). Kampf *et al.* reported that *S. aureus* carriage among hospital staff was approximately 33.8% (174). Moreover, rates of colonization were described to be higher among particular patient groups, e.g. HIV patients (257, 327), individuals with *S. aureus* skin infections and skin diseases (153, 390), particular with insulin-dependent diabetes (206), and patients undergoing hemodialysis (183, 211, 401) reaching up to 100% in individuals with atopic dermatitis (153, 245).
The carriage *status* has been associated to an increased risk for the development of staphylococcal infection (148, 185, 367, 386). Hospitalized patients are particularly at risk of developing a staphylococcal infection not only due to their increased *S. aureus* colonization rate, but also as a consequence of their general compromised immunity, and the use of invasive clinical procedures, such as surgery or the introduction of foreign indwelling medical devices, that serve as a port of entry of *S. aureus* into presumably sterile body sites.

The severity of *S. aureus* infections can range from minor to life-threatening, local to systemic and acute to chronic. *S. aureus* infections are categorized in three general types: 1. local infections - superficial lesions such wound infections, skin and soft tissue infections (SSTIs); 2. systemic and life-threatening - endocarditis, osteomyelitis, pneumonia, brain abscesses, meningitis, bacteremia and septicemia; and 3. toxinoses- toxic shock syndrome (TSS), staphylococcal scalded-skin syndrome and food poisoning (65).

Notably, *S. aureus* is one of the leading causes of nosocomial infections worldwide. A recent Europe-wide survey report (2011-2012) showed that among pathogens implicated in nosocomial infections, *S. aureus* (12.3%) come second only after *Escherichia coli* (15.9%), but before other important pathogens such as *Enterococcus* spp. (9.6%) and *Pseudomonas aeruginosa* (8.9%) (98). Moreover, the prevalence of hospital associated infections (HAI), reported as the percentage of

patients with at least one HAI over the total number of patients, ranged from 2.3% in Latvia to the highest prevalence registered in Portugal of 10.8% (98). As opposed to the majority of European countries, in Portugal, *S. aureus* takes the lead over *E. coli* as the most frequent cause of nosocomial infections (98).

Besides being one of the most important pathogens in healthcare settings, *S. aureus* is also an important agent of infections in immunocompetent individuals outside hospitals. *S. aureus* in the community have been described to cause mainly skin and soft tissue infections (SSTI), but also invasive infections such as bacteremia, necrotizing pneumonia and necrotizing fasciitis (73, 148).

RESERVOIRS AND TRANSMISSION ROUTES

The Centers for Disease Control and Prevention (CDC) estimated that for every 20 people hospitalized in the USA, one will develop a nosocomial infection (98). As the result of the high burden caused by *S. aureus* in hospitals, staphylococcal infections are the principal focus of infection control programs.

As mentioned before, *S. aureus* carriers are considered at higher risk for the development of staphylococcal nosocomial infections. About 15-67% of the nosocomial bacterial infections occur through patient-to-patient transmission (139, 383) by direct contact with colonized (or infected) patients. On the other hand, these infected or colonized patients can contaminate the surrounding environment, where bateria can persist up to a year (115, 315). Indirect contact through contaminated surfaces, are potential sources of re-infection of patients and colonization of healthcare workers (HCW), other patients or even patient's visitors. Moreover, healthcare workers uniforms should be regarded as an important vehicle of dissemination, since up to 60% of uniforms were described to be contaminated with potentially pathogenic bacteria, including drug-resistant ones, as reported in a study conducted in 2008 where a total of 135 HCW were enrolled (389). An example of this complex network of transmissions was described in a Portuguese neonatal intensive care unit NICU where HCWs, plastic folders protecting clinical files and also mothers' nipples were identified as potential reservoirs and/or vehicles of dissemination of *S. aureus* (55).
Healthcare infection control and prevention programs should be focused on the entire route of transmission as a whole, and not addressing each reservoir individually. This should be done by

improving active surveillance, mainly by screening patients and HCW, by implementing appropriate hand hygiene practices, using personal protective equipment and performing good environmental desinfection. Moreover, decolonization of patients and health-care workers has been proved efficient in reducing the risk of staphylococcal infection (29, 278, 339).

COST OF INFECTIONS AND INFECTION CONTROL

Hospital Infections have a substantial cost all over the world. The costs associated with treatment and control of *S. aureus* infections are approximately $14.5 billion per year in the USA (260). In 2005, the healthcare system of the USA spent an estimated value of $830 million to $9.7 billion with *S. aureus* infections (229). The infections associated with drug resistant *S. aureus* have an increased cost when compared to those susceptible, being a direct cost ($3,000 to $35,000) for the treatment of a single infection episode (62).

The decreasing of *S. aureus* frequency in invasive disease in many European countries is encouraging (97). Many of these countries adopted strict infection control measures, with good results. In the UK, since 2001, specific measures were applied in hospitals at the country level with an amazing success (89, 284). These included: 1) mandatory reporting of all *S. aureus* resistant bacteraemia, 2) public standardization of incidence rates, 3) guidelines for preventing hospital-associated infections, 4) establishment of a national hand hygiene campaign, 5) prudent use of antibiotics, and 6) the implementation of the so called 'high impact interventions', i.e. care "bundles" focusing on key clinical procedures that, when not appropriately performed, can increase the risk of infection. A decreasing trend of MRSA bacteraemia was clear from 2001 to 2009 in the UK, with a 62% reduction in the incidence of MRSA in blood cultures (158).

In Portugal, the prevalence of nosocomial infections increased from 9.3% in 1993 to 11.5% in 2012 (88), which parallels the increased burden of *S. aureus* in invasive disease. In the past, two national programs coordinated by Direcção Geral de Saúde (DGS) (www.dgs.pt) aimed to respond to this increasing problem, one created in 1998, the "National Program for Infection Control", and the other in 2008, the "National Program of Preventions of Resistance to Antimicrobials". But very recently (2013) the prevention of nosocomial infection was integrated as a national objective in the "National Program for the Prevention and Control of Infection and Antimicrobials Resistance" from DGS,

audited by the ECDC (European Centre for Disease Prevention and Control) (87). This program aims to normalize the mission and structure of the commissions of infection of all hospitals in Portugal in what regards to prevention and control of antimicrobial resistance. Moreover, the program intends to standardize the procedures in clinical practice through the implementation of an hospital "bundle" with the following rules: five moments of hand hygine accordingly to the World Health Organization (WHO) guidelines (http://www.who.int/gpsc/tools/Five_moments/en/), correct use of gloves, frequent cleaning of touch surfaces, and antimicrobial correct procedures. Finally, finantial incentives for good pratices related with infection control, antimicrobials resistance prevention and antibiotic consumption were implemented from 2013 on. No results on the impact of these measures are available yet.

ANTIMICROBIAL CONSUMPTION

The excessive use of antimicrobials is one of the main forces driving the development and spread of antimicrobial resistance. Antimicrobial resistance became a serious public health concern as the result of the emergence and spread of highly resistant bacteria and also due to the limited choice of antimicrobial agents to treat these pathogens.

Different actions to control the use of antimicrobials in the community and hospitals have been followed in the different European countries, as demonstrated by the consumption of antimicrobials report from the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) published by the European Centre for Disease Prevention and Control in 2010 (ECDC) (See Figure 1) (99). The consumption of antimicrobial for systemic use (ATC (anatomical therapeutic chemical) group J01: beta-lactams, penicillins; other beta-lactam antibacterials; tetracyclines; sulfonamides and trimethoprim; macrolides, lincosamides and streptogramins; quinolones; and others) in both hospitals and community is defined by number of DDD (defined daily dose) per 1 000 inhabitants and per day.

In hospitals, the consumption of antimicrobials for systemic use in 18 countries varied from as low as 1.1 to as high as 3.0 DDD per 1 000 inhabitants per days (See Figure 1). Based on this report, a low antimicrobial consumption was reported in Portugal (1.4 DDD per 1 000 inhabitants and per day) when compared to e.g. Italy, France and Finland (See Figure 1). The most frequent antimicrobial agent used in hospitals was penicillin, followed by cephalosporins and other beta-

lactams, quinolones and macrolides.

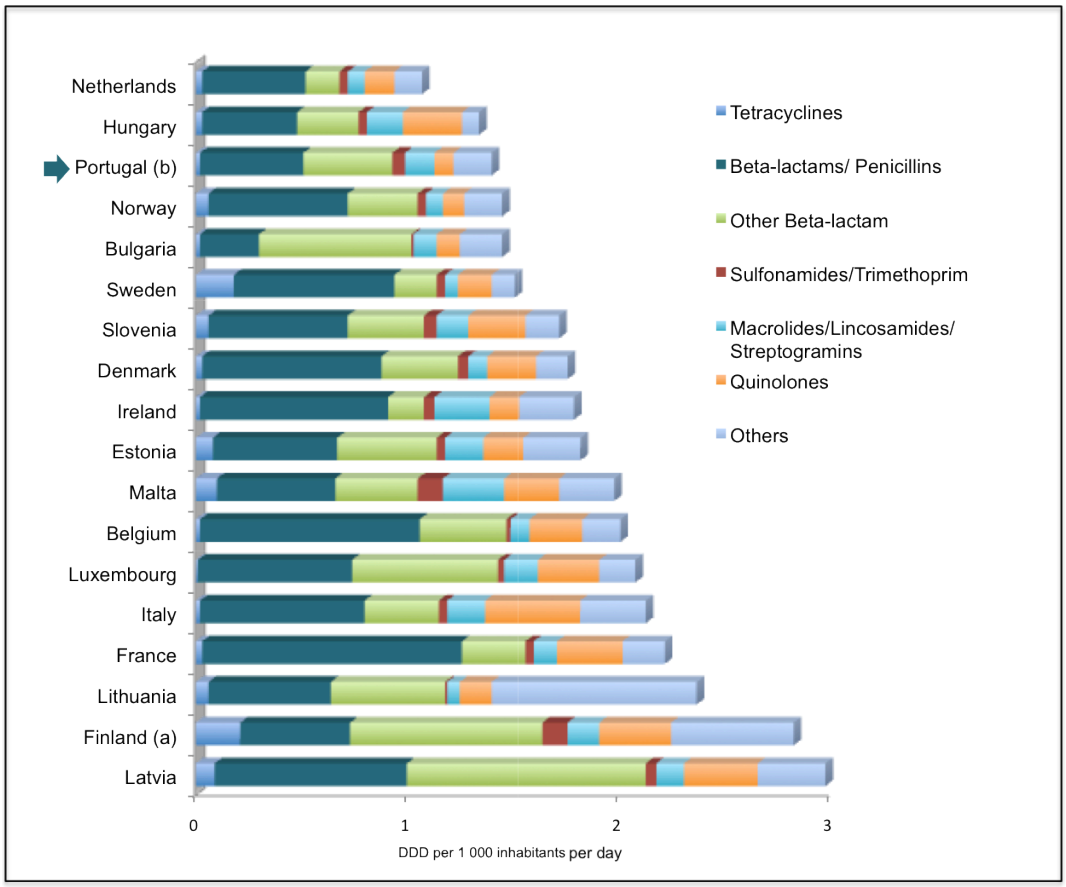


Figure 1. Distribution by country of antimicrobial consumption for systemic use in the hospital, in 18 countries (2010). DDD: defined daily dose; (a) Finland: data include consumption in remote primary health care centres and nursing homes; (b) Portugal: data correspond to public hospitals only [adapted from (99)].

The extensive consumption of antimicrobials in the clinical setting is not exclusive to hospitals. The proportion of antimicrobial consumption for systemic use in the community reported by 26 countries (See Figure 2) is even higher than that reported in the hospitals and varied from 11.1 DDD per 1 000 inhabitants per day, reported in Estonia, to 39.4 DDD per 1 000 inhabitants per day, in Greece (99).

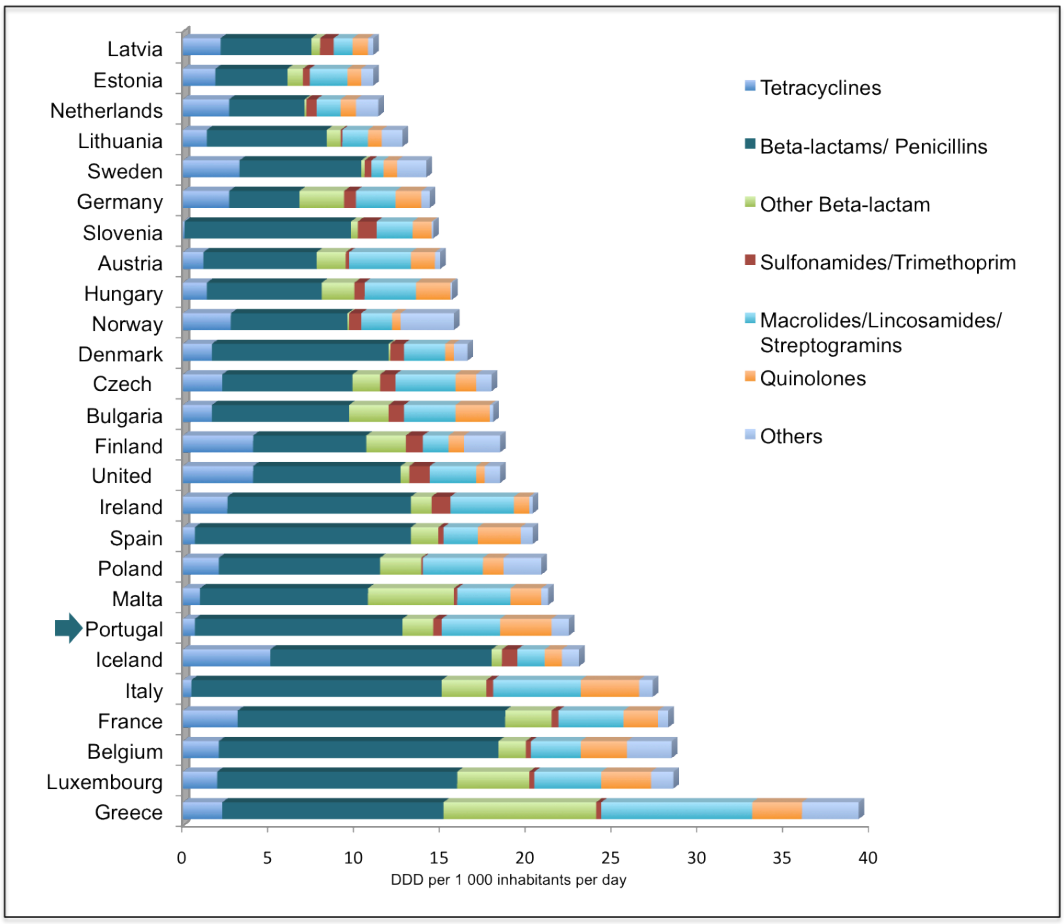


Figure 2. Distribution by country of antimicrobial consumption for systemic use in the community, in 26 countries (2010) [adapted from (99)].

The lowest consumption (< 16.7 DDD per 1 000 inhabitants and per day) was reported in the north of Europe, e.g. Scandinavian and Baltic countries and the highest (≥ 22.4 DDD per 1 000 inhabitants and per day) in the south of Europe e.g. Greece, Italy and also in Portugal with 22.4. In Portugal, the most commonly used antimicrobials were the combinations of penicillins and penicillins with extended-spectrum, followed by macrolides and tetracyclines. Portugal was the second European countries with the highest consumption of macrolides, just preceded by Italy.

ANTIMICROBIAL RESISTANCE

The major problem faced presently by clinicians when treating *S. aureus* infections is the fact that in the hospital environment these bacteria are resistant to multiple classes of antimicrobial agents. This situation has resulted from a history of 50 years of repeated adaptation of *S. aureus* to the

introduction of different antibiotics into clinical practice.

In the pre-antibiotic era, the mortality rate associated to *S. aureus* infections was above 80% (328). This scenario changed after the occasional discovery by Alexander Fleming in 1928 of penicillin (109), later referred to as the first antibiotic of the antibiotic era. With the introdution of penicillin into clinical practice, in the early 1940s, an effective treatment was provided in the treatment of bacterial infections for the first time in medical history, leading to a dramatic decrease in the mortality and morbility associated to *S. aureus* infections (41, 317). Despite its promising efficiency, ten years later, more than 90% (or virtually all) *S. aureus* became resistant to penicillin (184). Similarly to what was observed for penicillin, the development of new antibiotics and its application into clinical practice was consecutively followed within just a few years, by the emergence of resistance (See Figure 3).

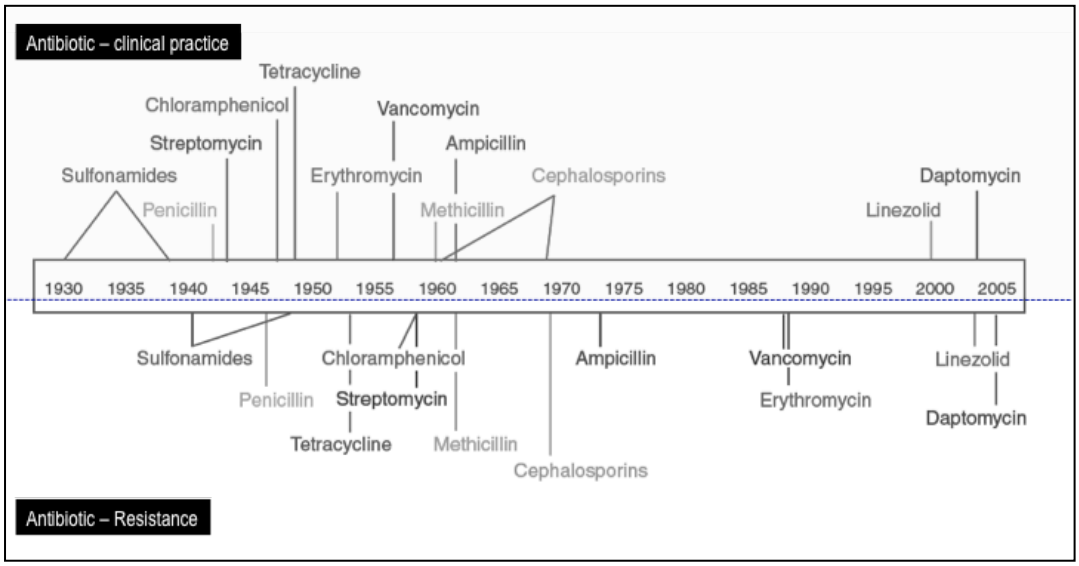


Figure 3. Timeline of antibiotic introduction in the clinical practice and the emergence of antibiotic resistance [adapted from (53)].

Antimicrobials target essential bacterial functions as cell wall synthesis (e.g. beta-lactams and glycopeptides), protein synthesis (e.g. aminoglycosides, tetracyclines, macrolides, lincosamides, chloramphenicol, mupirocin and fusidic acid), nucleic acid synthesis (e.g. quinolones), RNA synthesis (e.g. rifampin) and metabolic pathways such as folic acid metabolism (e.g. sulfonamides and trimethoprim) (See Table 1). The excessive use of antimicrobials along time usually induces the

development of resistance either by the emergence of point mutations or acquisition of foreign resistance genes; alteration of the antimicrobial target, degradation of the antimicrobial or redution of the antimicrobial concentration inside cell (See Table 1).

Table 1. Antimicrobial agent classes, mechanisms of action and resistance [adapted from (8, 348, 395)].

Antibiotic classes (examples)	**Mechanism action**	**Resistance type**	**Mechanism of resistance**
Beta-lactams (Penicillins, Cephalosporins, Carbapenems, Monobactams)	Cell wall synthesis: Bind to penicillin-binding proteins (PBPs) and inhibit the transpeptidation step in the peptidoglycan synthesis stimulate autolysins	Altered target site	Additional and altered PBPs
		Enzymatic degradation	Beta-lactamase
Glycopeptides (Vancomycin, Teicoplanin)	Cell wall synthesis: Inhibit transglycosylation and transpeptidation steps in peptidoglycan synthesis - bind to D-Ala-D-Ala	Altered target site	Altered peptidoglycan cross-link target
			Excess of peptidoglycan
		Target overproduction	
Aminoglycosides (Gentamicin, Trobamycin, Neomycin, Kanamycin, Streptomycin)	Protein synthesis: Inhibit 30S ribosomal subunits	Enzymatic modification (AMEs)	Phosphotransferase; Adenyltransferase; Acetyltransferase; Bifunctional enzyme
			Inactivation by aminoglycoside – modifying enzymes
		Decreased uptake	Changes in outer membrane permeability
Tetracyclines (Tetracycline, Tigecycline)	Protein synthesis: Inhibit 30S ribosomal subunits	Altered target	Production of proteins that bind to the ribosome and alter the conformation of the active site
	Disrupt bacterial membrane	Efflux	New membrane transporters
Macrolides/Lincosamides (Azithromycin, Erythromycin)	Protein synthesis: Inhibit 50S ribosomal subunits	Altered target	Methylation of ribosomal active site with reduced binding
		Efflux	Mef type pump
Chloramphenicol (Chloramphenicol)	Protein synthesis: Inhibit 50S ribosomal subunits	Enzymatic degradation	CAT
		Efflux	New membrane transporters
Mupirocin (Mupirocin)	Protein synthesis: Inhibit isoleucyl-tRNA synthetase	Target modification	
Fusidic acid (Fusidic Acid)	Protein synthesis: Inhibit protein synthesis (elongation factor G)	Altered target	Mutation leading to reduced binding to active site(s)
		Decreased permeability	Chloramphenicol acetyltransferase
Quinolones (Ciprofloxacin, Norfloxacin, Levofloxacin)	Nucleic acid synthesis Bind DNA gyrase	Altered target	Mutation leading to reduced binding to active site(s)
		Efflux	New membrane transporters
Rifampin (Rifampicin)	RNA synthesis Bind to beta-subunit of bacterial RNA polymerase	Altered target	Mutations leading to reduced binding to RNA polymerase
Sulfonamides/ Trimethoprim (Trimethoprim, Trimethoprim-Sulfamethoxazole)	Metabolic pathways folic acid metabolism Inhibit enzymes responsible for tetrahydrofolate production	Altered target	Mutation or recombination of genes encoding DHPS and DHFR
			Acquisition of new low-affinity DHFR genes
			Promoter mutation leading to overproduction of DHFR

Beta-lactam antibiotics: Methicillin

Among all classes of antibiotics, beta-lactams are considered the gold standard in the treatment of *S. aureus* infections. Beta-lactams act by acylation (inactivating) the active site of the transpeptidase of the penicillin-binding proteins (PBPs), preventing cell wall synthesis.

Resistance to beta-lactam antibiotics was first described for penicillin and was found to be associated to the presence of the *blaZ* gene. This gene can be transported in a plasmid or be chromosomally encoded and mediates the production of a beta-lactamase that acts by hydrolyzing the beta-lactam ring of penicillin.

In order to overcome the problem of the penicillin-resistant *S. aureus* infections, in 1959 a semisynthetic antibiotic, resistant to beta-lactamases, was developed - methicillin (originally called celbenine) (302). At that time methicillin was believed to be a definitive cure to *S. aureus* infections. However, within only two years (1961), in the UK, the first treatment failure was reported, and methicillin-resistant *S. aureus* (MRSA) emerged (170). Althought currently accumulating resistance to other semisynthetic beta-lactams, the designation of MRSA is still extensively used in opposition to MSSA (methicillin-susceptible *S. aureus*).

The main mechanism associated with methicillin resistance is related with the presence of an extra penicillin-binding protein (PBP), named PBP2A (147) with reduced affinity to methicillin and most beta-lactams antibiotics. Notwithstanding, other types of resistance to beta-lactams have been described in *S. aureus*, namely the overexpression of the beta-lactamase (118, 227), the presence of chromosomal mutations (16, 141, 253) or the overexpression of PBPs (352).

Mechanism of methicillin-resistance

In the presence of beta-lactams, transpeptidase domain of the four native PBPs (PBP1 to 4) of *S. aureus* is inactivated, and the extra PBP, PBP2A, a peptidoglycan transpeptidase, in cooperation with the transglycosylase domain of PBP2, catalyzes the cell wall biosynthesis (147, 333, 357). PBP2A is encoded by the *mecA* gene, which is carried by a heterologous mobile genetic element called staphylococcal cassette chromosome *mec* (SCC*mec*) (177). More recently, a *mecA* homologue – *mecC* - was described in a *S. aureus* strain of animal origin (LGA251) (180); the *mecC* gene has a 69% nucleotide identity with the typical *S. aureus mecA*. This *mecA* homologue originally named $mecA_{LGA251}$ and later called *mecC* confers lower levels of resistance to methicillin and cefoxitin than *mecA*, does not amplify with the typical *mecA* primers, and fails to agglutinate in

the PBP slide agglutination tests (119, 320). Since *mecC* first description, in 2011, several studies have reported the presence of this gene in different *S. aureus* genetic backgrounds of both animal and human origin (22, 82, 283), with limited geographic distribution in Europe (22).

SCC*mec* structure

SCC*mec* integrate into the staphylococcal chromosome at a specific site (*attB* or the integration site sequence ISS), within the 3' end of an open reading frame (*orfX*) coding for a methyltransferase of RlmH type, located near the origin of replication (30, 76, 150).

SCC*mec* elements are composed by i) *mec* gene complex, ii) *ccr* gene complex and iii) three flanking regions, the joining (J) regions. The *mec* gene complex, besides the *mecA* gene include also intact or truncated *mecA* regulators, *mecI* (repressor) and *mecR1* (sensor inducer). More recently, *mecR2* was also identified as a regulator of *mecA* through binding to the methicillin repressor *mecI* (11). To date five different *mec* complex classes (A, B, C1, C2 and E) have been described in *S. aureus* according to its structure (See Table 2) (76, 144, 163, 165, 168). The *ccr* complex encodes for recombinases of the invertase resolvase family responsible for mediation of site- and orientation-specific integration and excision of SCC*mec* from the chromosome (168, 177). A total of five *ccr* allotypes have been described: four allotypes containing combinations of *ccrA* and *ccrB* genes (*ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*); and another allotype containing a single gene, *ccrC* (See Table 2). The remaining part of the SCC*mec* is composed by the very heterogeneous J regions (previously called "junkyard" regions) (J1, J2, J3), containing non-essential components and genes conferring antibiotic and heavy metal resistance. The J1 region is located between the right junction and the *ccr* complex, the J2 region located between the *ccr* complex and the *mec* complex and J3 region extends from the *mec* complex to the *orfX*.

SCC*mec* is classified into types and subtypes. The SCC*mec* types results from the combination of the class of the *mec* gene complex and the allotype of the *ccr* gene complex and the variations in the J regions are used to define the SCC*mec* subtypes (237). Currently, eleven different types of *SCCmec* elements (types I-XI) were reported in *S. aureus*, ranging in size from 20.9 to 66.9 Kb (See Table 2). Furthermore, a total of 19 subtypes have been described, 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) (25, 51, 163, 167, 192, 215, 237, 272, 404).

Table 2. SCC*mec* types identified in *S. aureus* (I to XI).

SCC*mec* type	SCC*mec* size (Kb)	*ccr* gene complex (*ccr* genes)	*mec* gene complexes	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

Adapted from http://www.sccmec.org/Pages/SCC_TypesEN.html

In contrast to the SCC*mec* types IV, V, VI and VII encoding resistance to beta-lactams only, the SCC*mec* types I, II, III and VIII carry additional resistance determinants for other antibiotics and heavy metal. This is conferred by the presence of integrating plasmids, like pUB110 encoding for resistance to kanamycin, tobramycin and bleomycin, pT181 coding for tetracycline resistance and pI258 coding for resistance to mercury; and transposons such as *Tn*554 carrying the *ermA* gene, which is responsible for inducible macrolide, lincosamide and streptogramin (MLS) resistance (168). In a single event the acquisition by *S. aureus* of these SCC*mec* types, gives rise to a multidrug resistant (MDR) bacteria.

Non beta-lactam antibiotics: Vancomycin

Throughout the past years, antimicrobials commonly used to treat MRSA infections were vancomycin and teicoplanin, tetracyclines, clindamycin, quinolones and fusidic acid, or the more recently released antibiotics linezolid, daptomycin, tigecycline, telavancin and ceftaroline. However, MRSA have progressively become resistant to a number of antimicrobials, including clindamycin and tetracycline (143). Based on antimicrobial resistance surveillance in Europe, in 2012, that included 29 countries, a high percentage of resistance to fluoroquinolone (81%) was registered among MRSA in invasive disease (97). Resistance was also noticed, although in lower frequency,

to rifampicin (5.7%) and linezolid (0.2%).

Historically, glycopeptides, in particular vancomycin, has been considered the antibiotic of last resort and the gold standard antimicrobial for the treatment of serious MRSA infections. However, the occurrence of intermediate (VISA), and high level of resistance (VRSA) in *S. aureus* has already been reported since the last 1990s and early 2000, respectively.

The first VISA was reported almost 20 years ago in Japan in 1997, and several other strains were described afterwards (149) in the USA (321, 322, 331), China (407), India (133) and Europe, including Portugal (120).

On the other hand, in 2002, the first report of a fully vancomycin-resistant *S. aureus* was described in a renal dialysis patient in Michigan, USA (21). Since then, the dissemination of VRSA has been limited, with a total of 15 reported and confirmed cases, namely in the USA (n=13), Brazil (n=1) and also in Portugal (n=1) (110, 121, 233, 250, 287, 305, 323, 349, 382, 388). Although not spreading significantly worldwide, the increasing reports of VRSA should be seen as a warning.

The problem of *S. aureus* developing multiple antimicrobial resistance lead to the limitation of treatment options, emphasizing the urgent need for the development of novel therapeutic strategies to target this pathogen.

NEW THERAPEUTIC STRATEGIES

Nowadays, although antibiotic resistance is one of the highest public health concerns, there is a disinvestment of pharmaceutical companies in the development of new antimicrobials (113). Several approaches to induce a protective immunity against *S. aureus* have been made (291), however vaccine development has repeatedly failed (68). New approaches not based in the interference with bacterial cell growth and survival, but rather in virulence mechanisms of *S. aureus* have been developed. Compounds acting against biofilm formation (216) or targeting the alpha-hemolysin (Hla) and phenol-soluble modulins (PSMs), two important *S. aureus* virulence factors, are being evaluated as possible therapies. Moreover natural compounds, such as honey, has been also explored, demonstrating antimicrobial activity against MRSA forming biofilm (235).

2. Typing methods used to characterize *S. aureus*

Numerous phenotypic and genotypic typing methods have been developed in the past 50 years to characterize *S. aureus*. However, along time, phenotypic methods have been gradually replaced by molecular typing techniques, which have been shown to have more ability to distinguish the

epidemiological related and unrelated isolates and have been used in long-term and short-term *S. aureus* epidemiological studies.

Molecular typing methods can be divided in two types: “band-based‘ and “sequence-based”. The band-based methods are methods where the variations in nucleotide sequence are detected indirectly by primer-binding and/or retriction sites (e.g. Pulsed Field Gel Electrophoresis (PFGE)), in contrast to the sequence-based methods where the precise order of nucleotides is determined and DNA sequence variations evaluated (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 sequence-based methods have the advantage over the band-based typing methods of enabling data interchangeability and reproducibility in a much easier way (116).

The choice of the most appropriate typing method is highly dependent on the scientific question that needs to be addressed. Usually outbreak situations demand more discriminatory methods and global epidemiology less discriminatory methods. An excellent molecular typing method should have an adequate discriminatory power, be highly reproducible, easy to perform and to interpret, generate interchangeable data, be inexpensive and not time consuming (336, 362). However, since no single method meets all these criteria, a strategy based on the combination of different typing methods for the molecular characterization of *S. aureus* has been established. This includes the characterization of isolates by Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), *spa* typing and SCC*mec* typing, in the case of MRSA (4). In addition, high throughput whole genome sequencing (WGS) emerged recently as a useful tool to type *S. aureus*, having the highest discriminatory power possible (188, 399).

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

Among the band based typing techniques, PFGE has bee considered the "gold standard" method in the molecular characterization of *S. aureus* clones (226). PFGE is based on the restriction of the chromosomal DNA by a restriction enzyme that cuts infrequently the DNA; in the case of *S. aureus* the most frequently used enzyme is the SmaI (346). After restriction of total DNA around 10 to 30 fragments are generated, ranging from 10 to 700 Kb. These fragments are resolved in a special electrophoresis apparatus where the orientation and duration of the electric field is changed periodically (“pulsed”), rather than submitted to a constant field as in conventional electrophoresis.

The interpretation of the resultant band patterns can be performed visually or automatically, using diverse software programs able no only to interpret PFGE band pattern but also to perform phylogenetic analysis e.g. BioImage (BioImage Corp., Ann Arbor, MI), GelCompar (Applied Maths, Sint-Martens-Latem, Belgium) Molecular Analyst Fingerprinting Plus (Bio-Rad, Hercules, California, USA) and BioNumerics (Applied Maths Sint-Martens-Latem, Belgium).

PFGE detects the gain and loss of enzyme recognition sites, which are associated to large size variations in the chromosome, namely duplications, deletions and insertions. The high frequency of these events renders this technique especially suitable for short-term or local epidemiology (outbreak) and not adjusted for long-term molecular evolution (346).

In spite of its utility mainly for infection control situations, PFGE is technically demanding and time consuming. Moreover, data interpretation is complex, not reproducible, making it difficult to standardize and to exchange between laboratories (363).

STAPHYLOCOCCUS AUREUS PROTEIN A (*spa*) TYPING

Spa typing, which is based on the sequencing of the polymorphic X region of the *S. aureus* protein A gene was the first sequence-based typing method used in the characterization of *S. aureus* (114, 319). The polymorphic region is composed of a variable number of short repeats (24 bp), ranging from 1 to 23 repeat units. This diversity in the X region of the gene is attributed to deletions, duplications and also point mutations (173, 319).

Nowadays, the standardized international nomenclature established for *spa* typing is the RIDOM. An online database for *spa* types deposit was built in SpaServer database (http://spaserver.ridom.de), implemented by an European wide-network (SeqNet.org) that includes 60 laboratories from 29 European countries. The *spa* sequences are usually analyzed using the StaphType software (Ridom GmbH, Wurzburg, Germany), which is synchronized with the SpaServer database. To each different 24 bp repeats a number is assigned and a sequence type is attributed to each dfferent repeat profile (e.g. the *spa* type repeats succession 26-30-17-34-17-20-17-12-17-16 is defined as spa-type t001). Furthermore, the evolutionary relatedness between different *spa* types can be analyzed using the based upon repeat patterns (BURP) algorithm implemented in the StaphType software, which defines clusters or clonal complexes (CC).

Currently, *spa* typing is the most useful technique in the characterization of *S. aureus*, since it combines cost effectiveness, short time performance, high reproducibility and full portability (116).

Moreover, due to the repeat structure of X region, it simultaneously reveal micro- and macrovariations, enabling the use of *spa* typing in both local (outbreaks) and global epidemiological studies, in this case usually complementated with other typing methods (187, 270). Nevertheless, since *spa* typing is based on a single locus, it has a relatively low discriminatory power (337).

MULTILOCUS SEQUENCE TYPING (MLST)

Multilocus sequence typing was proposed for the first time in 1998 as a sequence-based method for the identification of clonal relatedness among bacteria (217). Two years later, MLST was described for molecular typing of *S. aureus* isolates by Enright and colleagues based on the sequencing of an internal region (450-500 bp) of seven housekeeping genes scattered over the chromosome [carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphatase acetyltransferase (*pta*), triosesphonate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*)) (102).

These sequences can then be submitted to a large public 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 (e.g. MLST profile 3-3-1-1-4-4-3 defined as ST8). The genetic relatedness between isolates can be obtained by comparison of allelic profiles using the eBURST (based upon related sequence types) algorithm (**http://saureus.mlst.net/eburst**) (108). The eBURST analysis clusters together closely related STs into MLST clonal complexes (MLST-CC), where the founder ST is the ST that became the most predominant and has diversified by point mutation or recombination producing other related STs, differing in one or up to seven loci. STs differing in a single locus are designated as single locus variant (SLV) (108). Nowadays, the third version of eBURSTv3 is available and more recently a similar tool, the goeBURST (global optimal eBURST) (**http://goeburst.phyloviz.net/**) was developed as an improvement of eBURST, with different display options and containing a different algorithm (Kruskal algorithm) aiming to solve the problem of finding the optimal forest based - starting with a forest of singleton trees (each ST is a tree), the optimal forest is built by iteratively selecting links connecting STs in different trees and with the highest number of SLVs (111).

MLST provides information about *S. aureus* lineage or genetic background, and has been used for long-term surveillance as well as evolutionary studies (102, 107). Like *spa* typing, MLST generates accurate and reproducible data, a consequence of the internationally standardized nomenclature

deposited in a curated and updated database. Notwithstanding, MLST is costly and time consuming and has a low discriminatory power resultant from the fact that only housekeeping genes are screened, making this technique appropriate for long-term epidemiological studies (337).

STAPHYLOCOCCAL CASSETTE CHROMOSOME MEC (SCC*mec*) TYPING

The staphylococcal cassette chromosome *mec* (SCC*mec*) typing is essential for MRSA typing, since presently clone definition for this microorganism is based on the combination of the genetic background as defined by MLST and SCC*mec* type.

Since the first description of SCC*mec* in 1999 (166), a considerable number of strategies have been developed along side with the new SCC*mec* types descriptions, attempting to determine the structure of these elements (31, 151, 186, 237, 271, 405). Nearly all strategies rely on multiplex PCR strategies, where SCC*mec* elements are classified into types and subtypes. The SCC*mec* types are defined based on the combination of the type of *ccr* gene complex and the class of the *mec* gene complex. The SCC*mec* subtypes are determined based on the polymorphisms found in J regions, within the same SCC*mec* type.

Oliveira and De Lencastre described the first multiplex PCR strategy developed to detected SCC*mec* types I to IV (271). Five years later, Milheirico *et al* developed an improved update of these multiplex PCR, where SCC*mec* type I to IV typing scheme was optimized and the detection of two additional SCC*mec* types V and VI was included (237). Almost simultaneously, Zhang *et al.* have described a complex method for SCC*mec* I to V typing and SCC*mec* IV subtyping (405) however the proposed approach was too laborious since four separate multiplex reactions were needed. Other multiplex strategies were developed namely by Hisata *et al*, Kondo *et al* and Boye *et al,* however none of these approaches fulfilled all requirements for an optimal typing scheme (31, 151, 186).

Nowadays, the multiplex strategy described by Milheirico *et al* is extensively used in the SCC*mec* typing of MRSA (237). Notwithstanding, this strategy does not include the determination of SCC*mec* types VII to XI, or other variants that may be identified in the future, evidencing the need of constant updating of typing schemes.

The increased importance of SCC*mec* type IV during the last years due to the emergence of MRSA in the community has increased the need for the classification of the variants within this type of

cassette. Therefore, a multiplex PCR assay was developed to distinguish between SCC*mec* types IVa to IVh, based on the PCR amplification of six specific loci within the J1 region (237).

More recently, a nomenclature for SCC*mec* types was proposed by the IWG-SCC (International Working Group on the Staphylococcal Cassette Chromosome elements) (163) (http://www.sccmec.org/Pages/SCC_HomeEN.html), 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 an SCC*mec* harboring a type 1 *ccr* gene complex and a class B *mec* gene complex).

CLONE DEFINITION

MRSA clone definition, recommended nowadays by HARMONY the International Union of Microbiology Societies' European Staphylococcal typing network, a society that was created with the aim of normalizing and standardizing *S. aureus* typing methods, is based on the combination of MLST and SCC*mec* typing as a reference typing system (60). Accordingly, MRSA clones have been nowadays commonly defined based on its "original name", according to the geographic location they were firstly isolated, together with the combination of genetic background and SCC*mec* type (MLST-SCC*mec*) e.g. Brazilian clone (ST239-III) (86, 103, 299). However, in particular cases, the inclusion of additional molecular information like the *spa* type or specific virulence markers, as ACME and PVL, may help to facilitate the definition of clones.

WHOLE GENOME SEQUENCING (WGS)

Conventional typing methods have been successfully used for *S. aureus* epidemiological studies, but they provide a limited discriminatory power. In whole genome sequencing (WGS) analysis the complete nucleotide sequence is determined, with maximum resolution, at a single nucleotide level. The general workflow of sequencing is based on sample preparation, DNA sequencing, sequence assembly and bioinformatics analysis.

First generation sequencing was developed in 1977 by Frederick Sanger and was based on capillary electrophoresis. Almost 30 years after (2004), the Sanger sequencing was replaced by high-throughput sequencing, the second generation or next generation sequencing (NGS). NGS parallels the sequencing process producing thousands or millions of sequences, with much lower costs and increasing capacity, when compared to Sanger sequencing. Different sequencing

platforms are currently available, namely Illumina, Roche 454 and SOLiD. More recently, the first bench sequencers were developed, including Illumina MiSeq, Ion Torrent Personal Genome Machine and the GridION mainly for use in the clinical environment. After sequencing, the sequence data reads are assembled *de novo* or assembled against a reference sequence, into contiguous DNA sequences (contigs).

As a research tool, WGS already demonstrated feasibility in staphylococcal studies, providing insights into the emergence, spread, evolution and dynamics of *S. aureus* (135, 224, 252, 290, 373, 399), showing sufficient discriminatory power to reconstruct intercontinental and local transmission of MRSA lineages (146) and also predicting antimicrobial resistance (188) and disease phenotypes (223, 399). Moreover, WGS was also used with clinical proposes, in the analysis of MRSA outbreaks (146, 188).

WGS is becoming less expensive, with lower sample preparation time and provides a massive amount of data. Although, nowadays specialized bioinformaticians are still necessary for data interpretation, friendly software is being developed that will allow to extract information, like ST, *spa* type, SCC*mec* type and presence of antimicrobial resistance and virulence genes in an almost authomatic way.

3. Molecular epidemiology of *S. aureus*

3.1. Methicillin-susceptible *S. aureus* (MSSA)

MSSA as commensal and pathogen

Over the years, most of the epidemiological studies in *S. aureus* have been focused on MRSA because of their high pathogenic potential and complexity of clinical treatment. The molecular epidemiology of methicillin-susceptible *S. aureus* (MSSA) has been less studied despite MSSA being frequently found as cause of infections both in the healthcare setting (69, 94, 264, 282) and in the community (83, 351).

MSSA in hospitals and community

Until the mid 1990s, MSSA were a common cause of both serious and uncomplicated *S. aureus* infections among healthy individuals in the community, whereas MRSA were confined to the hospital setting. The epidemiology of infection changed in the late 1990s when MRSA emerged in the community as a causative agent of skin and soft tissue infections (SSTIs) in otherwise healthy

individuals, becoming in some cases endemic and more prevalent than MSSA, as observed in the USA (112, 181, 248). Of interest, in the USA, in the era of epidemic CA-MRSA, the MSSA played a reversed role relative to MRSA, where MSSA assumed the role of a nosocomial pathogen and CA-MRSA was predominant cause of infections in the community (69). This same discrepancy was observed in the USA, in recent study conducted in five academic medical centers, where MSSA in invasive disease account for 14.3% of all *S. aureus* comparing with 10.2% of MRSA (71).

In Europe, the frequency of MSSA in invasive disease in hospitals increased from 2005 to 2008 (3.4% per year; 95% CI 3.0–3.7) (74), calling the clinicians attention for the importance of MSSA as a pathogen. In a Spanish Hospital, MSSA accounted for over 67.9% of the *S. aureus* isolates (13).

Relatedness of MSSA from hospital and community

Evolutionary studies have identified genetic relationships between MSSA with hospital and community origin (102, 194). In Portugal, no significant differences were observed in the distribution of the MSSA established in the community and in the hospitals, with 16 out of 20 clones found in both settings, including the four major clones (CC30, CC45, CC5 and CC8) (3). In agreement with this, several genetic backgrounds found in young healthy carriers in the community in Spain were also identified in different hospitals (12, 13). In this study, in both environments, CC5 was clearly predominant followed by CC30, CC45, CC8 and CC15.

MSSA and PVL

The prevalence of MSSA carrying Panton Valentine leukocidin (PVL) in invasive disease varies in different countries (137)(http://www.spatialepidemiology.net/srl-maps/). An alarming high prevalence of PVL (>35%) was described among MSSA clinical isolates in several African countries, like Cape Verde Islands, Cameroon, Madagascar, Morocco, Niger, and Senegal (6, 34). The prevalence of MSSA carrying PVL in the remaining countries appears to be variable. In a Spanish hospital PVL prevalence increased from 0% in 2002 to 36.4% in 2006/2007 (13, 66, 286). In Portugal, MSSA carrying PVL showed a high frequency (34.2%) associated with SSTIs in children attending a pediatric emergency department of a central hospital in Lisbon (57). But in USA and France, among SSTIs, the PVL positive MSSA showed a wide range of prevalence ranging from 12% to 93% (202, 213, 248, 274).

MSSA were found to cause mild infections as SSTIs, but also severe forms of necrotizing

pneumonia in otherwise healthy children and young adults in the community (124, 202, 350). The clinical relevance of MSSA in the community appears to be strongly associated with the presence of PVL. But like in the hospital environment, the prevalence of community associated infections caused by MSSA carrying PVL can vary widely, raging from 2% to 17% (e.g. USA and China) (159, 202, 274, 343).

Concerning MSSA colonization in the community, the prevalence of MSSA carrying PVL was relatively low 0.5-1.5% in the USA and China (191, 232) in comparison with the high prevalence (>35%) described in Africa, namely Cape Verde (6) and Mali (309) and in Indonesia (16%) (83).

MSSA genetic diversity

S. aureus population in humans consists of about ten dominant lineages and several minor lineages. The dominant human lineages of *S. aureus* are often referred to by their MLST clonal complex (CC). They are CC1, CC5, CC8, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 (107).

Although only a limited number of clonal types among the MSSA population gave rise to the worldwide epidemic MRSA clones, through SCC*mec* acquisition, no clear information exist on the real frequency of SCC*mec* acquisition by MSSA (42, 84, 103). It is now established that the population structure of MSSA is genetically more diverse than that of MRSA (137), although the precise extension of this diversity is uncertain. Few data exist on the population structure of MSSA, their relationship with MRSA and associated virulence factors.

Despite the high genetic diversity of the MSSA, four major clonal complexes seem to be predominant in the USA and Europe, although with different significance, namely CC30, CC5, CC8 and CC45 (3, 12, 13, 236, 296) indicating the presence of pandemic MSSA lineages, described long time ago (84, 103, 130). On the other hand, different predominant MSSA lineages were reported in Asia (CC45, CC25, CC121, CC59 and CC188) (45) and Africa (CC1, CC15, CC30, CC121 and CC152) (34).

In Portugal, only a study focusing in MSSA population was performed; the results showed that for almost 15 years (1992 to 2001), the four major MSSA clones were ST30-t012 and ST34-t166 (CC30), ST5-t002 (CC5) and ST45-t330 (CC45) in both, hospital and community settings (3).

Relatedness of MSSA and MRSA

Due to their epidemiological importance, MSSA has been extensively compared with MRSA. Goering and colleagues have shown in a global clinical trial in 10 countries that a considerable overlap exist between MSSA and MRSA genetic backgrounds of the 292 isolates obtained from skin infections in the community (127). Moreover, David *et al* demonstrated that the overlap in the MLST genotypes of MSSA and MRSA of both community and hospital origin, from patients seeking care for infections in the USA, fell into five STs: ST5, ST8, ST1, ST30 and ST59 (69) all common MRSA genetic backgrounds in various regions of the world. In the United States, several studies addressed the relationship between circulating MRSA and MSSA associated with colonization (40, 213, 347), SSTIs (213, 240), and infections among children (244). In all studies a considerable overlap was observed between MSSA and MRSA populations. As recently demonstrated by Miko *et al* in a study where invasive and SSTIs MSSA isolates from reference laboratories from each USA Census region, the two most prevalent MSSA clones (ST5-t002 and ST8-t008) found were related with the most two predominant MRSA clones found nowadays in the USA (236). In Portugal, the MSSA population recovered from infection and colonization in both community and hospital settings (ST30, ST34, ST5, and ST45) was scarcely or not related with the MRSA population found at the same time period in the Hospitals (ST239, ST247, ST22, ST5) (3). The same scenario of lack of relatedness between MSSA and MRSA was observed in Africa (34). In Cameroon, Madagascar, Morocco, Niger and Senegal, 58% of MSSA isolates from infection belonged to five major MSSA clones CC121 (ST121-t314), CC15 (ST15-t084), CC1 (ST1851(SLVST1)-t127) and CC152(ST152-t355) (34), where none was related with the most successful MRSA clones found in the same time period, particularly ST239/241-III. Also in Taiwan, most MSSA lineages (ST1, ST6, ST7, ST12, ST188, and ST97) have a different genetic background when compared with the local (44, 45, 379) and worldwide MRSA (127). The only common genetic background found among MSSA and MRSA was ST59, related with an epidemic community-associated MRSA clone found in Taiwan (44, 379).

MSSA and MRSA virulence

There is a longstanding debate about whether MRSA is more virulent than MSSA. Some epidemiological studies have shown that MRSA are more virulent than MSSA (62, 197). Recently, a report on the prevalence of pneumonia among hospitalized children in Hawaii revealed that patients infected with MRSA more frequently developed pulmonary complications than MSSA patients (197).

Also, longer periods of hospitalization were described in children with osteomyelitis resultant from MRSA with community origin, when compared with osteomyelitis caused by MSSA (222). Moreover, higher mortality rates were associated with MRSA infections when comparing with MSSA (62, 67, 387). However, the opposite was also reported, where MSSA was associated to more severe infections than MRSA or, on the other hand, where no significant difference in virulence between the two was observed. David and colleagues suggested possible "reversal role" of MSSA, where in transplant patients from a large academic hospital in the USA, was observed that MSSA more frequently was associated with bacteremia, endocarditis, and sepsis than MRSA (69). Also Kaplan and colleageous, in a three-year surveillance at the Texas Children's Hospital, documented a higher percentage of MSSA (8.2%) than MRSA (4.4%) in invasive disease (175).

MSSA population structure overtime

Few studies reported MSSA population structure over time. A structured survey was performed where population structure and distribution of PVL positive MSSA and evolutionary history over time was assessed. The MSSA isolates were selected from the collection of the French National Reference Center for Staphylococci representing five continents (19 countries) over nearly 3 decades (from 1981 through 2007) (293). In France, the MSSA population changed drastically from 1981 through 2007. Until 2000, MSSA isolates ST80 were absent, and from 2001–2005 emerged and accounted for 15.6% of isolates collected, afterwards during 2006–2007, ST80 became the most frequent ST (27.8%). Conversely, ST121 accounted for 56.3% of isolates collected before 1990 and decreased to 30.8% in 1991–2000, 13.3% in 2001–2005, and to 0% after 2005. In another study, Nulens and colleagues reported between 1999 and 2006 the presence of an endemic MSSA population (CC5, CC8, CC22, CC30, and CC45) in invasive disease in a Belgium Hospital (264). In Portugal only one study addressed the evolutionary history of MSSA along almost a decade (1992-2001), where the authors observed a stable predominance of CC30, CC5, CC45, and CC8 over time (3).

Continuous surveillance of MSSA is important for the understanding of the dynamics of MSSA population, and to anticipate the emergence of MRSA clones, both in the community and in the hospital.

3.2. Methicillin-resistant *S. aureus* (MRSA)

Hospital-associated (HA)-MRSA

Prevalence and trends

Healthcare-associated (HA) infections have been defined as infections developed in the hospitals (122, 154). Accordingly, HA-MRSA are typically defined as MRSA causing infections in inpatients or in individuals that were not colonized or infected with MRSA at hospital admission and that developed infection after 48h or more of hospitalization (http://www.cdc.gov/mrsa/).

Between 1997 and 2003 SENTRY Antimicrobial Surveillance Program reported that the worldwide HA-MRSA prevalence varied from as high as 67% in Japan to rates of 36% in the USA, 29% in Latin America, 23% in Australia, and 23% in Europe (24, 90, 243). Based on the last report (2012) from the European Antimicrobial Resistance Surveillance Network (EARS-Net) (97), the actual MRSA prevalence in Europe in invasive disease is about 17.8%. Notably, HA-MRSA prevalence varies broadly from country to country, ranging from lower than 5% in northern European countries [Sweden (0.7%), Norway, Netherlands and Denmark (1.3% each), Iceland (1.7%) and Finland (2.1%)] to 53.8% and 53.9 in Portugal and Romania, respectively.

The HA-MRSA trends in Europe for the period 2009–2012 indicate that MRSA are decreasing in most European countries, as observed in Belgium, Croatia, France, Germany, Hungary, Ireland and the UK. However, in other countries such as Portugal, Poland, and Romania MRSA prevalence increasead significantly during this period. The decreasing (or stabilizing) tendency is consistent with what has been reported in recent years from European national surveillance programmes and local reports (74, 172). This positive trend may be seen with caution since the prevalence of MRSA is above 25% in almost one third of countries, particularly in Southern and Eastern European countries (Romania, Portugal, Malta, Greece, Cyprus, Italy and Poland).

In Portugal, HA-MRSA prevalence in invasive disease has increased over time, from 31.9% in 2001 to 53.8% in 2012 (97). Two MRSA decreasing prevalence periods in Portugal were registered, one in 2008-2009 from 52.9% to 49.1% and the other, more recently, in 2011-2012 from 54.6% to 53.8%, however these decreases were not statistically significant. Moreover, a national report from Direcção Geral de Saúde (DGS, Portugal) from 2012, including 43 Portuguese hospitals from which a subset of isolates were included in the European project EARS-Net/ECDC, denote even higher MRSA rates, where among the 243 *S. aureus* causing hospital associated infections, 73.7% were

MRSA (88).

On the other hand, in the USA, MRSA in invasive infections have also become a serious problem, where a continuous increase in prevalence over a 10-year period (1998-2007) from 32.7% to 53.8% was reported (234).

MRSA origin and evolution

The origin and evolution of MRSA has been debated for long. The relationship between the first MRSA and successive MRSA clones was first explained based on the "single clone theory", where the authors suggested that the extant MRSA clones have a common MRSA ancestor, resultant from single SCC*mec* introduction event into an MSSA (189). Later, the "multiclone theory" was proposed, which is nowadays the most widely accepted, where pandemic MRSA clones were suggested to have emerged from the SCC*mec* introduction into several distinct MSSA precursor lineages (103, 130, 263). This was clearly shown by Enright and colleagues while analyzing *S. aureus* recovered in 20 countries, between 1961 and 1999, where the authors found that different *S. aureus* clones were associated to a specific genetic background-SCC*mec* element combination (103). This study estimated that MRSA has emerged at least 20 times as a result of *de novo* SCC*mec* acquisition (263, 298).

MRSA have shown to be higly clonal, represented by a limited number of human-associated lineages, the CC1, CC5, CC8, CC9, CC12, CC15, CC22, CC25, CC30, CC45 and CC51 (107). Among these, five main lineages CC5, CC8, CC22, CC30 and CC45 are the most successful, and have become disseminated worldwide in the hospitals settings (103, 273, 298).

Molecular epidemiology of HA-MRSA

Some authors described the evolutionary history of *S. aureus* as waves of antimicrobial resistance. The first wave occurred in the mid 1940s, with the emergence of penicillin-resistant *S. aureus* [(20, 182) cited by (42)]. By the early 1950s, the penicillin-resistant *S. aureus,* represented by phage type 80/81 clone, become pandemic causing infections in both hospitals and community [(307) cited by (78)]. Pandemic phage type 80/81 largely disappeared nowadays, but the prevalence of the penicillinase-producing *S. aureus* has remained very high.

The second wave occurred when the first MRSA was isolated in a patient at Colindale Hospital (London, UK) in 1961, only two years after the introduction of methicillin, a semisynthetic beta-

lactamase resistant penicillin developed to treat penicillin-resistant *S. aureus* infections (170). This was a hallmark in *S. aureus* epidemiology, and the beginning of a major global public health concern – the "MRSA era". This first hospital-associated (HA-MRSA), was the archetypal COL strain, belonging to the so-called Archaic clone (ST250-I) (75, 103) which is part of CC8. The Archaic clone was described until the 1970s circulating in hospitals throughout Europe (64), but was seen rarely in the rest of the world, and in the 1980s virtually disappeared (138). This event marked the end of the second and the beginning of the third wave of antibiotic resistance.

In the third wave, descendants of the archaic MRSA clone, belonging to CC8 emerged, e.g. the Iberian clone (ST247-IA) reported in Spain in 1989 (95), the Brazilian clone (ST239-IIIA) described in Brazil in 1992 (64, 345), and the Portuguese clone (ST239-IIIvar) widely spread in Portugal in the mid 1980s and early 1990s (5) (See Table 4). The international scenario was dominated by this CC8 epidemic HA-MRSA clones until the end of 1990s.

This was followed by a period where other highly successful MRSA clones belonging to other genetic backgrounds emerged, including some of the nowadays most dominant worldwide MRSA pandemic clones (see Table 3): i) the New York/Japan clone (NY/JP) (ST5-II) and the Paediatric clone (ST5-VI/IV) belonging to CC5, described in USA in 1998 (226) and in Portugal in 1992 (311), respectively; ii) the EMRSA-15 clone (ST22-IVh) belonging to CC22, in the UK in 1993 (171) ; iii) the EMRSA-16 clone (ST36-II) belonging to CC30, described in the UK in 1993 (171) and iii) the Berlin clone (ST45-IV) belonging to CC45, first reported in Germany in 1993 (381, 391).

Table 3. Molecular characterization and geographical distribution of the major HA-MRSA [adapted from (38, 85, 86)].

	Epidemic Clones	CC	ST-SCC*mec*	*spa* Type	Geographical Spread
HA-MRSA	**COL**, **Archaic**	**8**	8	t008, t009, t194	Colindale (UK), Uga, Can, US, Ast, Den, Ger, Swi, UK
	Iberian, EMRSA-5, Rome		ST247-I	t008, t051, t052, t054, t200	US, Isr, Aus, Bel, Cro, Cze, Den, Fin, Fra, Ger, Hun, Ita, Net, Nor, Pol, Ger, Slo, Spa, Swe, Swi, UK
	Brazilian, **Hungarian**		ST239-III(A)	t030, t037, t234, t387, t388	Alg, Arg, Bra, Can, Chi, Par, Uru, US, Chn, Ind, Ids, Kor, Mon, RoG, Rus, Saud, Sin, Sri, Tai, Tha, Vie, Ast, Aus, Cze, Den, Fin, Ger, Gre, Hun, Net, Nor, Pol, Ger, Slo, Spa, Swe, UK
	New York/Japan (NY/JP) or USA100	**5**	ST5-II	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Can, Mex, Uru, US, Chn, Isr, Jap, Kor, SA, Saud, Sin, Tai, Ast, Bel, Den, Fin, Fra, Ger, Hun, Ire, Nor, Ger, Swe, Swi, UK
	Paediatric or USA800		ST5-IV/ V	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Arg, Par, Jap, Tai, Den, Ger, Nor, Pol, Ger, Slo, UK, Alg, Arg, Bra, Col, Uru, US, Kor, Tai, Ast, Aus, Den, Fin, Fra, Ger, Gre, Nor, Pol, Ger, Spa, Swe, UK
	VISA, hVISA		ST5-II VISA/hVISA-II	t002	Jap, Chi, Braz, Por
	EMRSA-15	**22**	ST22-IVh	t005, t022, t032, t223, t309, t310, t417, t420	Can, Chn, Kuw, Sin, Ast, NZ, Aus, Bel, Cze, Den, Fin, Ger, Hun, Ire, Mal, Nor, Ger, Spa, Swe, UK
	EMRSA-16 or USA200	**30**	ST36-IV	t018, t253, t418, t419	Can, Mex, US, Ast, Aus, Bel, Den, Fin, Ger, Gre, Ire, Nor, Ger, Spa, Swe, Swi, UK
	Berlin or USA600	**45**	ST45-IV	t004, t015, t026, t031, t038, t050, t065, t204, t230, t390	Arm, Chn, Isr, US, Ast, Aus, Bel, Den, Fin, Ger, Hun, Net, Nor, Spa, Swe, Swi

Africa: Alg- Algeria; Gab- Gabon; SA- South Africa; Uga- Uganda. The Americas: Arg- Argentina; Bra- Brazil; Can- Canada; Chi- Chile; Col- Colombia; Mex- Mexico; Par- Paraguay; Uru- Uruguay. Asia: Arm- Armenia; Chn- China; Est- Estonia; Ind- India; Ids- Indonesia; Isr- Israel; Jap- Japan; Jor- Jordan; Kor- Korea; Kuw- Kuwait; Lat- Latvia; Leb- Lebanon; Mon- Mongolia; RoG- Republic of Georgia; Rus- Russia; Saud- Saudi Arabia; Sin- Singapore; Sri- Sri Lanka; Tai- Taiwan; Tha- Thailand; Vie- Vietnam; Oceania: Ast- Australia, NZ- New Zealand. Europe: Aus- Austria; Bel- Belgium; Bul- Bulgaria; Cro- Croatia; Cyp- Cyprus; Cze- Czech Republic; Den- Denmark; Fin- Finland; Fra- France; Ger- Germany; Gre- Greece; Hun- Hungary; Ice- Iceland; Ire- Ireland; Ita- Italy; Mal- Malta; Net- Netherlands; Nor- Norway; Pol- Poland; Por- Portugal; Rom- Romania; SR- Slovak Republic; Slo- Slovenia; Spa- Spain; Swe- Sweden; Swi- Switzerland. CC: clonal complex; HA: hospital acquired; MRSA: methicillin-resistant Staphylococcus aureus; ST: sequence types; SCC*mec*: Staphylococcal Cassette Chromosome *mec*. Spa types obtained using the Ridom.

Besides the existence of these worldwide globally distributed pandemic HA-MRSA epidemic clones, there are descriptions of variants of these epidemic clones or less predominant clones.

Clonal replacement overtime

The occurrence of clonal replacement of MRSA clones has been reported in many countries. A dramatic clonal replacement occurred in Hungary about ten years ago when the NY/JP and South-German clones almost completely replaced the Hungarian clone (56), which was later gradually replaced by EMRSA-15 (157). Another study conducted in Spain demonstrated that between 1998 and 2002 the Iberian clone was replaced by EMRSA-16 (285). Moreover, a study performed in a small geographic region in Switzerland showed that the NY/JP, Berlin, Southern German and Iberian clones were present over a period of eight years, however the relative proportion of the four clones was different along time and in each hospital (28). Recently, a study performed in Italy by Campanile *et al* observed the clonal replacement of MRSA clones over a 17-years period where the Iberian and Brazilian clones were replaced by the Southern German clone that was then displaced by the EMRSA-15 (37).

In Portugal, MRSA clonal replacement in hospitals was also reported (7, 9, 10). The HA-MRSA population structure has been extensively characterized, and four successive clonal waves were reported (7). Early in 1992/93 the widespread Portuguese clone was replaced by the Iberian clone that was displaced by the MDR Brazilian clone (1994/95), that subsequently disseminated all over the country (7). In the year 2000, the Brazilian clone was replaced by the epidemic EMRSA-15 clone, which is still the most predominant clone accounting for 72 to 75% of all HA-MRSA isolates (7, 104). Soon after the emergence of EMRSA-15, the NY/JP clone or variant of this clone (ST105-II) emerged as the second most predominant (7, 104).

The reasons lying behind the existence of these cycles of HA-MRSA clones that emerge, spread and then decline in the hospital settings are still unclear. The antibiotic selective pressure probably is one of the most important factors in the modulation of the HA-MRSA dynamics. In particular the emergence of ciprofloxacin (fluoroquinolone) resistance, was demonstrated to be key for the replacement of the Hungarian clone by the EMRSA-15 and the perpetuation of this clone in the Hungarian Hospitals (157).

HA-MRSA outside hospitals

In addition, several reports of HA-MRSA clones causing infections outside hospitals have been published. The EMRSA-15 clone considered one of most successful hospital-associated clones, is also seen frequently disseminated in the community. Moreover, EMRSA-15 has been found to adapt easily to different hosts, being found also in companion animals and wild animals (54, 70, 210). EMRSA-15 was found in colonization and infection in the community in different geographic regions, namely in Italy (219), Belgium (368) and UK (156). In Portugal, EMRSA-15 was found colonizing dogs (54) and also as a contaminant of the handrails of public buses in Oporto (26% of the sampled buses with MRSA) (324) and Lisbon (36.2% of the sampled buses with MRSA) (58).

Moreover, in Japan, the NY/JP clone was reported in the community among healthy children and pediatric outpatients, in episodes of necrotizing pneumonia (280). In East Asia, HA-MRSA ST239-III and ST5-II clones have spread from the hospitals into the community (332). Moreover, cases of community-associated necrotizing pneumonia have been also found associated with a variant of the HA-MRSA ST5-II (179), and this clone was also found in public transports in Japan (179). Curiously, the imposed biological cost associated with larger size cassettes, SCC*mec* type II and SCC*mec* type III, did not restricted these HA-MRSA clones from spreading into the community, where probably much lower antimicrobials exist.

Community-associated (CA)-MRSA

The perception of MRSA regarded as a strictly nosocomial pathogen changed greatly during the last two decades. Although the majority of the MRSA infections are still registered in the hospitals, since the beginning of the 90s, MRSA has been increasingly recognized as a cause of infections also in the community (CA-MRSA) (131, 148, 355).

CA-MRSA definition

CA-MRSA infections are defined by the Centers for Diseases Control and Prevention (CDC) by a set of epidemiological criteria (http://www.cdc.gov/): individuals likely to have a CA-MRSA infection if MRSA is isolated in an outpatient or inpatient within 48 hours after hospital admission; moreover individuals should not have a medical history of MRSA infection or colonization in the past year and no history of admission to a nursing home, nursing facility, or hospice, dialysis, surgery, permanent indwelling catheters or foreign medical devices within this same period.

Although the CDC epidemiological definition is widely accepted, it is only based on the patient's

epidemiological data and bacteria's phenotypic and genotypic data are not taken into account. Phenotypic and genotypic features that characterize CA-MRSA include non–multidrug resistance profile (resistance to less than 3 classes of antibiotics besides beta-lactams), lower MIC values for oxacillin (around 32 lg/ml or less) or imipenem (around 1 lg/ ml or less) when compared with HA-MRSA (oxacillin: 128 lg/ml or imipenem: 32 lg/ml), specific genetic backgrounds, carriage of the small size cassette SCC*mec* IV/V and PVL (265, 341, 371).

If in the beginning of the 1990s CA-MRSA were completely distinct from HA-MRSA based on epidemiological, phenotypic and genotypic features, presently this is no longer valid (72). CA-MRSA have conquered the hospital environment in some countries, such as USA, where they have acquired resistance to multiple antibiotics (169, 316). Moreover, traditional HA-MRSA clones like NY/JP and EMRSA-15 have already been described in the community (155, 179, 219). Due to these changes in MRSA epidemiology, probably the best definition is the one based in a combination of epidemiological, phenotypic and genotypic data, as already suggested (238, 276).

Populations at risk

MRSA have been emerging worldwide as a leading cause of severe infections in particular closed and crowded populations with privileged close contact. This includes children in day care centers (242), athletes (268, 294), prisoners (220), military personnel (101, 408) intravenous drug users (275) and men who have sex with men (91) with median ages of 23 years old (254).

CA-MRSA colonization rates

CA-MRSA nasal and skin colonization constitutes a predisposing factor for infection in healthy individuals, and increase dissemination through skin-to-skin contact. Although few large-scale studies exist about CA-MRSA colonization, dispersed reports demonstrated that the CA-MRSA colonization rates varied among different populations, geographic locations and between different body sites. A wide range of CA-MRSA nasal colonization rates is usually observed in high-risk populations: in the general pediatric population ranged from 0.4%-9.2% (63, 117, 162, 255) in children attending daycare centers from 3-24% (1), in athletes from 1-25% (136); and men who have sex with men from 2.2-25.7% (91). However, in the remaining population the carriage rate are generally low. In the USA where CA-MRSA infections are now epidemic, a large study performed between 2001 and 2004 revealed a low prevalence of CA-MRSA nasal carriage in the healthy

population (1.5%) (132). Low rates of CA-MRSA nasal carriage were also reported in the UK (less than 1%)(140), in Japan (3.7-4.3%) (151, 280) and in Taiwan (1.9-11.6%) (160, 208). In the same way, early studies performed in Portugal indicated an extremely low prevalence of CA-MRSA carriage (<1 %) in the nasopharynx of young children attending daycare centers (1999, 2009) (310, 344) and in the anterior nares of military draftees, nonmedical university students, and high-school students (1999) (310).

Although CA-MRSA nasal carriage rate is generally low, other body sites appear to have higher colonization rates. Among patients with CA-MRSA SSTI, different patterns of body colonization was observed with 25% of patients colonized in the nares followed by 17% of patients colonized in the inguinal area, 13% in the rectal area and only 6% in the axilla (397). These observations suggest that other not yet explored reservoirs of CA-MRSA might exist and should be screened in surveillance studies.

CA-MRSA prevalence of infections

The most common infections caused by CA-MRSA are skin and soft-tissue infections (SSTIs) (70–90%), although more serious invasive infections, such as necrotizing pneumonia, necrotizing fasciitis, bacteremia and septic shock or even death have been reported (193, 202, 207). The burden of SSTIs varies geographically, in France from 2000 to 2003 only 1–3% of all SSTIs were caused by CA-MRSA (77, 297) whereas in Portugal CA-MRSA was associated with 7.9% of children's with SSTIs attending the pediatric emergency (57). On the other hand, a study conducted in the emergency departments, in USA, during one month of sampling (2004), showed that approximately 60% of SSTIs were caused by CA-MRSA (249).

Regarding other type of infections, CA-MRSA accounted for 2.4% of severe pneumonia in adults hospitalized in 12 university emergency departments during the winter-spring of 2006 and 2007 (247), and to severe pneumonia with a high mortality (30%), in the case of co-infection with influenza (142, 292, 308). Cases of fatal pediatric necrotizing pneumonia were reported e.g. in the USA, Japan and France (39, 124, 164). Also, cases of severe necrotizing fasciitis caused by CA-MRSA have been described as well in the USA from 2003-2004 (241).

CA-MRSA origin and evolution

The origin of CA-MRSA was somehow a controversial issue in the beginning of the CA-MRSA era. If by one hand some authors suggested that CA-MRSA may have originated from nosocomial MRSA that escaped to the community (2), on the other hand, other authors suggested that the small size SCC*mec* (SCC*mec* IV or V) was acquired by different MSSA epidemic clones in the community (CC1, CC8, CC30, CC59 and CC80) (32, 269, 353). In the beginning of CA-MRSA period, five major CA-MRSA clones carrying PVL were recognized (See Table 4).

Table 4. Molecular characterization and geographical distribution of the major CA-MRSA and LA-MRSA clones [adapted from (38, 85, 86)].

	Epidemic Clones	CC	ST-SCC*mec*	*spa* Type	Geographical Spread
CA-MRSA	**USA300** pvl+/ACME+	**8**	ST8-IVa	t008, t024, t064, t190, t206, t211	SA, Gab, Can, US, Est, Ind, Isr, Jap, Jor, Leb, NZ, Aus, Bel, Bul, Cro, Cze, Den, Fin, Fra, Ger, Hun, Ice, Ita, Net, Nor, Pol, SR, Spa, Swe, Swi, UK
CA-MRSA	**USA400** pvl+	**1**	ST1-IVa	t127, t128, t174, t175, t176, t386, t558	SA, US, Chn, Ids, Lat, Leb, Tai, NZ, Aus, Bel, Cro, Cyp, Den, Fin, Fra, Ger, Ice, Net, Nor, Pol, Rom, Spa, Swe, Swi, UK
CA-MRSA	**Southwest Pacific** pvl+ or USA1100	**30**	ST30-IVc	t012, t018, t019, t021, t138, t268, t276, t318, t338, t391	SA, Can, US, Jor, Lat, Leb, NZ, Aus, Bel, Cyp, Cze, Den, Fin, Fra, Ger, Ice, Ita, Net, Nor, Pol, Spa, Swe, Swi, UK
CA-MRSA	**Taiwan** pvl+ or USA1000	**59**	ST59-IVa/V/VII	t199, t216, t444	US, NZ, Aus, Bel, Cze, Den, Fra, Ger, Ice, Net, Nor, Pol, Spa, Swe, Swi, UK
CA-MRSA	**European** pvl+	**80**	ST80-IVc	t044, t131, t376, t416, t436, t455, t1109	Jor, Leb, Aus, Bel, Bul, Cro, Cyp, Cze, Den, Fin, Fra, Ger, Hun, Ice, Ita, Net, Nor, Spa, Swe, Swi, UK
LA-MRSA	**ST398** pvl+(LA-MRSA)	**398**	ST398-IVa/V/VII like	t011, t034, t108	Bel, Chi, Den, Ger, Hong Kong, Ita, Net, Aus, Bel, Can, Nor, Por, Spa

Africa: Alg- Algeria; Gab- Gabon; SA- South Africa; Uga- Uganda. The Americas: Arg- Argentina; Bra- Brazil; Can- Canada; Chi- Chile; Col- Colombia; Mex- Mexico; Par- Paraguay; Uru- Uruguay. Asia: Arm- Armenia; Chn- China; Est- Estonia; Ind- India; Ids- Indonesia; Isr- Israel; Jap- Japan; Jor- Jordan; Kor- Korea; Kuw- Kuwait; Lat- Latvia; Leb- Lebanon; Mon- Mongolia; RoG- Republic of Georgia; Rus- Russia; Saud- Saudi Arabia; Sin- Singapore; Sri- Sri Lanka; Tai- Taiwan; Tha- Thailand; Vie- Vietnam; Oceania: Ast- Australia, NZ- New Zealand. Europe: Aus- Austria; Bel- Belgium; Bul- Bulgaria; Cro- Croatia; Cyp- Cyprus; Cze- Czech Republic; Den- Denmark; Fin- Finland; Fra- France; Ger- Germany; Gre- Greece; Hun- Hungary; Ice- Iceland; Ire- Ireland; Ita- Italy; Mal- Malta; Net- Netherlands; Nor- Norway; Pol- Poland; Por- Portugal; Rom- Romania; SR- Slovak Republic; Slo- Slovenia; Spa- Spain; Swe- Sweden; Swi- Switzerland. CA: community associated; LA: livestock-associated; CC: clonal complex; MRSA: methicillin-resistant *Staphylococcus aureus*; ST: sequence types; SCC*mec*: staphylococcal cassette chromosome *mec*. Spa types obtained using the Ridom

These clones were described as having emerged in different geographic areas (79, 371). The first CA-MRSA infections were reported in Western Australia in 1993 among Pacific Islander population without predisposing risk factors for healthcare contacts, from remote areas. These isolates belonged to the so-called Southwest Pacific clone (SWP) (ST30-IVc, PVL) (61), which is a modern descendent of the early CC30 phage type 80/81 clone (MSSA, PVL positive, penicillin resistant) and is nowadays disseminated worldwide (300).

Nearly after (1997/1999), in the USA, four pediatric deaths caused by sepsis or necrotizing pneumonia called the attention of the medical community to CA-MRSA. These deaths were associated to isolates belonging to the clone, later known as USA400 (ST1-IVa, PVL), belonging to the CC1 (18, 39). Afterwards a new CA-MRSA clone was identified in the USA, the USA300 (ST8-IVa, PVL) belonging to CC8, that completely replaced the USA400 clone (72, 248). USA300 is nowadays the leading cause of SSTIs in the community in the USA where it became endemic (239, 403), and has invaded the hospitals, displacing the classical HA-MRSA clones, being considered a major public heath concern (169, 316). Subsequently, USA300 clone spread worldwide and has been described in virtually all countries (259, 303).

In Europe, the first CA-MRSA outbreaks were reported in Greece, being caused by isolates belonging to a different clonal type, the so-called European clone (ST80-IVc/V), that was first described in 2003 by Aires-de-Sousa *et al*, corresponding to strains dated back to 1993 and 1998 to 2000 (2). This clone is thought to have emerged originally in the Mediterranean, Middle East, or North Africa, since the first patients infected in Europe were described to have recently travelled to these regions (81, 196, 218). Moreover, particularly in some of these regions (sub-Saharan Africa), MSSA strains belonging to ST80 were found (59, 293, 318), raising the question about the origin of the European clone. Fusidic acid resistance mediated by *fusA*, *fusB*, or *fusC* genes, is a characteristic of this clone (195, 371). Since the first description, several reports of infection associated with the European clone were registered in many European countries, namely in France and Switzerland (371), Belgium (81), Germany ((392), Austria (190), The Netherlands (334, 380), Norway (145), Finland (176), Denmark (106), Sweden (105) but also in North Africa (Algeria) (23) and the Middle East (355). In contrast to the recognizable spread of USA300 clone into the USA hospitals, in Europe, nosocomial outbreaks due to the European clone have only been sporadically reported (2, 50, 203). These may indicate that the European clone seem less well adapted to persist in hospital compared with USA300 (277, 358).

In Asia, another CA-MRSA clone was decribed more or less simultaneously - the Taiwan clone (ST59-IVa/V_T, PVL) (33, 377). The Taiwan clone was described in the beginning to carry a unique SCC*mec* structure, SCC*mec* V (initially designated as SCC*mec* VII) (342). Since the first identification, the Taiwan clone was immediately described as MDR (including resistance to tetracycline, erythromycin, clindamycin, streptomycin, kanamycin and chloramphenicol), in contrast to the typical CA-MRSA antimicrobial susceptibility profile (33, 342). Nowadays, this clone is still the most predominant in Taiwan (160) and has also been isolated in Hong Kong (152) and in the USA (USA1000) (348).

In the early 2000, in Europe, a livestock-associated (LA)-MRSA lineage assigned to CC398 was described causing infections mainly in individuals in contact with animals, in France and The Netherlands (14, 374) (See Table 4). The ST398 clone (ST398-IVa, V and VII-like) was first identified in colonization of livestock and pig farmers with close livestock contact (14), however descriptions of infections in individuals without risk factors for livestock contacts were also reported (372, 394). Since the early reports, ST398 clone has been especially described in the northern European countries, and particularly associated with livestock (209). In some parts of The Netherlands ST398 clone accounted for up to 25% of the total MRSA (364), but ST398 was also described in Germany, Belgium, Italy, Austria, Spain, the United States, Canada and Australia (129, 329, 364, 394). ST398 MRSA isolates are mainly associated to pigs, but they were already isolated in other animal species such as dogs, horses, cattle, and poultry (330, 394). Colonization and transmission is believed to primarily occur between animals, however, pigs were also described as important reservoirs for human colonization and infection (361). The secondary human-to-human transmission seems infrequent and with lower disease burden (100, 361, 398). A human adapted variant of ST398 clone (ST398-t571) was recently reported as a major agent of severe infections in the USA (New York City), Canada, France, Dominican Republic and Belgium (290, 356, 365, 369). This ST398 human-associated lineage form the most ancestral ST398 clade, showed a high transmissibility capacity between humans, contrasting with the limited transmissibility of LA ST398 (290, 356). An additional signal of adaptation of this clone to humans is the acquisition of ST398 isolates of PVL, already reported in The Netherlands, Sweden, UK and China (335, 361, 384, 400).

Several lines of evidence suggest that the epidemiology of CA-MRSA have changed during the past 15 years, where besides the extensive worldwide distribution of the five main CA-MRSA epidemic

clones (259, 303), the entry into the hospitals already took place resulting in the emergence of multidrug resistant CA-MRSA (332) and the descriptions of variants of the prototypes clones or to the emergence of new and less epidemic clones have been reported (303).

In Portugal, no structured survey was ever performed, and based on dispersed reports, different CA-MRSA genetic backgrounds were found. Isolates belonging to CA-MRSA genetic backgrounds were sporadically reported in the hospital settings including ST121-IV (10), ST8-IV, ST1-IV, ST82-IV, ST88-IV and ST82-IV (7), European clone (ST80-IV) (57) and USA300 clone (256). Moreover, the less epidemic clones ST82-IV, ST939-IV (SLV ST72) and ST931-IV (SLV ST8) were found colonizing the nasopharynx of young children attending daycare centers (310, 344). Also, MRSA belonging to ST398 clone was isolated in pigs and in a nasal swab from a veterinarian (288, 289).

CA-MRSA virulence

CA-MRSA has been associated with an enhanced virulence, when compared to HA-MRSA. The higher pathogenic potential of CA-MRSA is believed to be due to the presence of CA-MRSA specific virulence factors, but also to the higher expression of the traditional MRSA virulence factors (36, 92). One of the factors that has been epidemiologically linked to CA-MRSA virulence is the PVL, a two-component leukocidin (LukS and LukF) that targets the immune system cells, such as polymorphonuclear neutrophils (PMNs), monocytes, and macrophages (92, 338). However, PVL is absent in many CA-MRSA isolates, leading to the recognition that PVL alone is not responsible for the enhanced CA-MRSA virulence, and also that PVL is a poor genetic marker for CA-MRSA (306, 406).

Another element that has been associated to an increased epidemicity of CA-MRSA, particularly in USA300 clone, is the the arginine catabolic mobile element (ACME), a pseudo-SCC like element, that contains two characteristic gene clusters, the *arc* operon that encodes several enzymes involved in the arginine deiminase catabolic pathway, and the *opp3* operon that encodes an oligopeptide permease system, which is believed to promote CA-MRSA higher transmissibility and survival (93).

Also, CA-MRSA virulence has been largerly associated with enhanced expression of the peptides phenol soluble modulins (PSMs) and the alpha-hemolysin both with cytolytic activity (92, 198, 279, 378).

4. Virulence factors in *S. aureus*

Besides the extraordinary capability to accumulate antibiomicrobial resistance determinants, *S. aureus* possess an enormous arsenal of virulence factors showing a unique ability to evade the host immune defenses. The ability of *S. aureus* to cause disease depends on multiple strategies and on the redundance of virulence factors.

4.1 Host-pathogen infection process

The pathogenicity of *S. aureus* results from a multitude of virulence strategies that allows an effective colonization, invasion and evasion of the host innate immune system (See Table 5).

Colonization

S. aureus is potentially adhesive, and this attachment capability is mediated by the interactions of the adhesins with host cells (skin, mucosa, endothelial cell surface), extracellular cellular matrix (collagen, fibronectin and fibrinogen) and plasma proteins, allowing an efficient colonization (43). *S. aureus* adhesins are cell wall anchored proteins belonging to a single family designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (52) which interact with host proteins in the extracellular matrix such as fibrinogen(225), collagen, fibronectin, vitronectin and elastin (52). MSCRAMMs most commonly found among *S. aureus* are elastin-binding protein S (EbpS), collagen-binding protein (Coa), fibronectin-binding proteins A and B (FnBPA, FnBPB), fibrinogen-binding proteins C, D and E (SdrC, SdrD, and SdrE) and the clumping factors A and B (ClfA, ClfB) [(43) cited by (360)]. MSCRAMMs besides being key for cell structural metabolism and host cell binding have been implicated in host cell internalization, host immune evasion, bacterial aggregation and biofilm formation (199, 326). Most of MSCRAMMs are encoded in the core genome, therefore are characteristically stable and inherited by vertical transfer. For this reason, the success of MRSA clones has not been in general linked to a specific MSCRAMM. The unique exception is the recently described adhesin, SasX, associated with the exceptional colonization and infection ability of MRSA ST239 clone, though *sasX* is encoded on a MGE (199).

Table 5. *S. aureus* virulence factors involved in the host infection process [adapted from (402)].

	Name	Abbreviation	Function
Virulence factors: **adhesion to host cells (MSCRAMM)**	Fibronectin binding proteins A and B	FnBPA, B	Adhesins for fibrinogen (FnBPA only), fibronectin and elastin
	Collagen-binding adhesin	Cna	Adhesin for collagen (type I and IV)
	Serin-aspartate repeat proteins C, D and E	Sdrs	Adhesins
	Bone sialo protein-binding protein	Bbp	Adhesin for bone sialoprotein (SdrE allelic variant), binds fibrinogen
	Elastin-binding protein	EbpS	Transmembrane adhesin for elastin and tropoelastin
	Polysaccharide intercellular adhesin	PIA	Adhesin for aggregation; involved in biofilm formation
	Iron-regulated surface determinant A	IsdA	Binds fibronectin, fibrinogen, transferrin, hemoglobin, hemin and fetuin
	Iron-regulated surface determinant B	IsdB	Binds hemoglobin and hemin
	Iron-regulated surface determinant C	IsdC	Binds hemin
	Iron-regulated surface determinant H	IsdH	Binds haptoglobulin and haptoglobulin–hemoglobin complex
	S. aureus surface proteins	Sass	Bind to the extracellular matrix
	S. aureus surface proteinG	SasG	Binds to the extracellular matrix; involved in biofilm formation
	Serine-rich surface protein	SraP	Binds platelets
	Extracellular matrix protein-binding protein	Emp	Binds extracellular matrix of host cells;involved in biofilm formation
	Extracellular adherence protein	Eap/Map	Impairs angiogenesis and wound healing; stimulates production of TNF alpha; and IL-6
	Emp homologue	Ebh	Binds extracellular matrix of host cells
	Plasmin-sensitive protein	Pls	Binds lipids of host cells; adhesion to nasal epithelial cells
	Second immunoglobulin-binding protein	Sbi	Binds Fc domain of immunoglobulin; binds complement protein C3 and promotes C3-C3b conversion
	Von Wille brand factor binding protein	vWbp	Binds and activates prothrombin; binds fibrinogen and vWfactor
	Extracellular adherence protein	Eap/Map	MHC-II analog protein; adhesion to *S.aureus* cells and host cells; involved in biofilm formation
virulence factors: **toxins, enzymes and other proteins**	beta-toxin	Hlb	Sphingomyelinase with cytolytic activity
	alpha-toxin	Hla	Cytolytic pore-forming toxin
	Leukocidins D, E and M	LukD/E/M	Kill leukocytes; bi-component pore-forming leukotoxins
	Phenol Soluble Modulins	PSMs	Pore-forming toxins or detergent activity
	Exfoliative toxins A, B and D	ETA/B/D	Exotoxins with superantigen activity; gluamate-specific serine proteases that digest desmoglein 1
	Enterotoxins	SEs	Gastroenteric toxicity; immunomodulation via superantigen activity
	Toxic shock syndrome toxin-1	TSST1	Endothelial toxicity (direct and cytokine-mediated); superantigen activity
	Coagulase	Coa	Binds and activates prothrombin; promotes conversion of fibrinogen to fibrin
	V8 protease	–	Serine protease
	Glycerol este rhydrolases	lip,geh,beh,	Triacyl glycerols degradation
	Fatty acid-modifying enzyme	FAME	Fatty acids modification
	O-acetyltransferase	OatA	Peptidoglycan O-acetylation
	Ptd Ins-phospholipase C	Plc	Phosphotidyl inositol-specific lipase activity
	Enolase	Eno	Catalyzes phosphor-glycerate to phosphoenol-pyruvate; binds to laminin
	Arginine Catabolic Mobile Element (3types)	ACMEI/-II/-III	Unclear role (aids colonization); seems to contain several enzyme and proteins (arginine deaminase system, oligopeptide permease, zinc-containing alcohol
	FPR-like 1 inhibitory protein	FLIPr	Binds formyl peptide receptor
S. aureus innate immune evasion: neutrophil migration and lysis	Staphylococcal superantigen-like 5	SSL5	Specific binding to P-selecting lycoprotein ligand-1 blocking PMN rolling
	Staphylococcal superantigen-like 11	SSL11	Binds to chemokine receptors
	Staphylococcal superantigen-like 1	SSL10	Binds to chemokine receptors
	Chemotaxis inhibitory protein	CHIPS	Blocks C5 a receptor and formyl peptide receptors
	Staphylococcal superantigen-like 7	SSL7	Binds to the Fc region of IgA and block recognition by neutrophils
	gama-toxin	Hlg	Bicomponent leukocidin; hemolysis
	delta-toxin	Hld	Cytolytic toxin; binds neutrophils and monocytes
	Panton-Valentine leukocidin	PVL	Bicomponent leukocidin; pore-forming toxin; kills leukocytes
	Leukocidins A and B (other name H and G)	LukAB/-HG	Bi-component pore-forming leukotoxin that kills PMNs

Invasion (intracellular life style)

Only recently, *S. aureus* was recognized as a facultative intracellular pathogen (314, 325). This capacity enables *S. aureus* to be protected against the attack from extracellular host defense. *S. aureus* can persist in a semi-dormant state known as small colony variants (SCV), where virulence expression is shutdown, which renders *S. aureus* intrinsically resistant to host attack and antibiotic therapy (314). The fibronectin-binding proteins (FnBPs) promote *S. aureus* attachment to the host-cell surface, and subsequent internalization to professional and nonprofessional phagocytes (325, 375). The exact mechanism of how and when SCV decide for the intracellular lifestyle remains to be determined.

4.2 Global regulators

The *S. aureus* pathogenesis process involves an array of surface and secreted proteins that are coordinately expressed at an appropriate time during the different stages of infection. Virulence expression is regulated in a growth phase dependent manner. Surface proteins are expressed in the exponential phase of growth, enabling the bacteria to attach to host cells, and the secreted proteins expressed in the late exponetial/stationary phase, allowing *S. aureus* to evade host in an efficient fashion (212).

The coordinated expression of these virulence factors is controlled by global regulatory elements, including: 1) two component regulatory systems (TCRs) where the accessory gene regulator (Agr) and the *S. aureus* exoprotein (SaeRS) are the most relevant (15, 46, 125, 261); and 2) transcriptional global regulators such as the staphylococcal accessory regulator (SarA) (48) and SarA homologues such as Rot (231, 312) but also SarT SarS, SarR, SarU, SarV, SarX, SarZ MgrA and TeaR (15, 214, 301).

The extensive range of *S. aureus* diseases results from the activation of this intricate regulatory network created by the activation of these regulators, in order to respond to environmental stimulus.

4.3 Alpha-hemolysin

Although *S. aureus* makes use of a myriad of toxins in the process of pathogenesis, only a small number have been associated with enhanced virulence. Among these, *S. aureus* alpha-haemolysin also known as alpha-toxin (Hla) is certainly the most relevant and intensively studied virulence factor. The alpha-hemolysin was initially named based on its lytic properties of red blood cells, but

several studies suggested a greater complexity of alpha-hemolysin action.

Alpha-hemolysin and disease mechanism

Alpha-hemolysin is a cytolytic and cytotoxic toxin implicated in severe skin infections, pneumonia and sepsis (26). Moreover, there are evidences that alpha-haemolysin is involved in intracellular survival, through the induction of endosomal escaping following phagocytosis .

Hla main target are the erythrocytes, altthough Hla was proven to target other human cells, including epithelial cells, endothelial cells, and an array of other hematopoietic-lineage cells including lymphocytes, monocytes and macrophages (26).

Alpha-hemolysin structure

The gene coding for alpha-haemolysin was discovered in the early 1980s (134, 178) and is present in the *S. aureus* genome in a single copy. The *hla* locus encodes Hla as a water-soluble monomer of 33.2 kDa, containing 293 amino acids (134, 354). This monomer forms heptameric units on the cellular membrane, as a beta-barrel transmembrane pore, with an internal diameter of about 1-2 nm (134, 354, 376) and a mushroom like shape, consisting of three domains (See Figure 4): A. **cap**: important for oligomerization and pore formation, is an hydrophilic domain composed by beta-sandwich structures, held together by the seven amino latches from seven monomers; B. **rim**: involved in membrane binding, works as membrane anchor to membrane bilayer and forms a three-strand beta-sheet under the cap domain. The aromatic and cationic aminoacid rich residues present between the cap and rim domains are involved in interactions with phospholipid head groups of target cell membranes; C. **stem**: composed of a 14 anti-parallel beta-barrel strands, forming the effective transmembrane channel (246, 376).

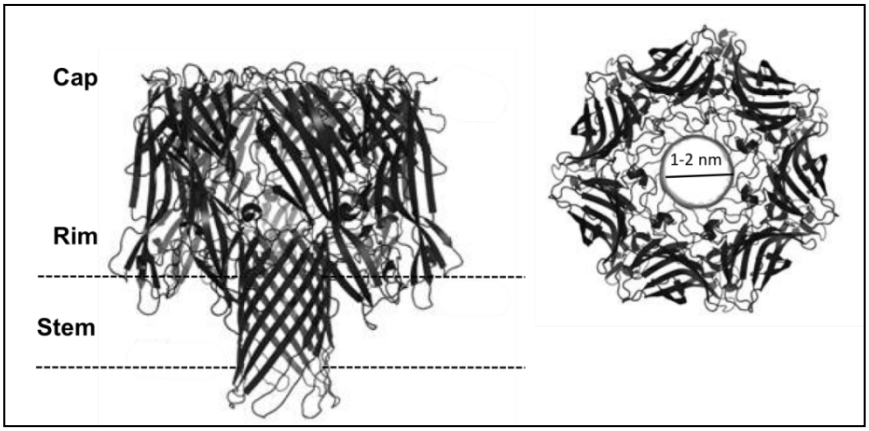


Figure 4. Crystal structure of *S. aureus* alpha-hemolysin derived from the RCSB Protein Data Bank (PDB, 7AHL) and modeled using PyMOL v.1.6. Cap: hydrophilic domain; Rim: membrane interface region; Stem: transmembrane channel

The toxicity of Hla results from the formation of the heptameric pore, leading to the alterations in ion gradients, loss of cytoplasmatic contents and activation of stress-signaling pathways resulting in cell lysis (27). The process of pore formation includes three steps: 1. binding of alpha-hemolysin monomers to membrane receptor (if is the case) of the susceptible (or non-susceptible) cells, followed by 2. heptamerization of the monomers into a prepore complex and, finally, 3.conformational changes of the prepore into a functional and mature transmembrane pore (359).

The *hla* locus is believed to be higly conserved across *S. aureus*, although genetic diversity was already described, where a non-synonymous SNP resulted in a premature stop codon in the *hla* among the hospital-associated EMRSA-16 clone. This mutation distinguishes the EMRSA-16 clone carrying the truncated Hla from the CA-MRSA South West Pacific clone and the phage type 80/81, all belonging to the CC30 (78). The evolutionary analysis of CC30 showed that the EMRSA-16 clone has evolved along time toward a niche-adaptation (hospital settings) to favor the colonization capacity in detriment of virulence, and the loss of Hla function might be related to this evolutionary trend (228).

Hla regulation

Similar to what is observed for most of the other *S. aureus* secreted proteins, alpha-hemolysin is not expressed constitutively. The *hla* expression is activated during the post-exponential-early stationary phase of growth, and toxin production is coordinately controlled by several regulators,

including the Agr, SaeRS, SarA, ArlS and SarZ (19, 126, 128, 201, 370). On the other way, alpha-hemolysin is downregulated by SarA homologues as Rot and SarT (230, 231, 313).

The primary and main regulation of *hla* is made by the Agr TCS, in particular by the RNAIII (See Figure 5). The RNAIII is the main effector molecule of the *agr* system containing the dual function of upregulation of secreted virulence factors as alpha-hemolysins (35, 96, 262) and downregulation of surface proteins (96, 161, 370). The RNAIII regulatory function is made through direct binding to the *hla* mRNA and indirectly through regulation of other regulators targeting virulence toxins, mainly at transcription but also translation level (47, 262, 295, 396) (See Figure 5).

On the other hand, alpha-hemolysin can have an Agr independent regulation, through e.g. the SaeRS, where the expression of *hla* result directly from the response regulator SaeR, one of the two-component systems encoded by SaeRS (125, 126, 301, 396). Besides the SaeRS, the SarA also positively affects the *hla* expression by either agr-dependent or *agr*-independent pathways, through SarT repression (49, 128, 313) (See Figure 5).

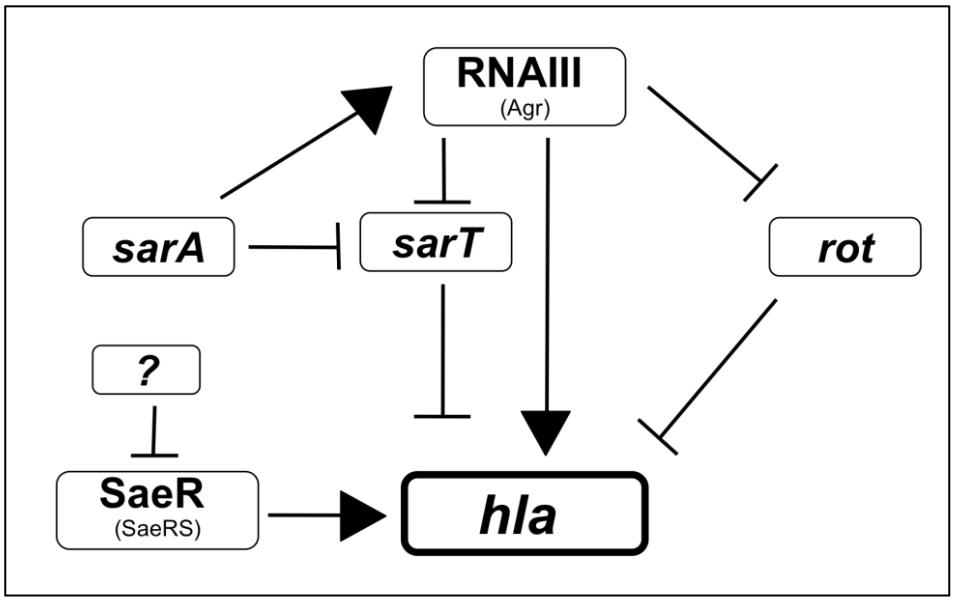


Figure 5. Schematic overview of the most probable regulatory interactions involved in the *hla* expression. The arrows indicate stimulation and the bars indicate repression. (?) unknown repressor of SaeRS system.

Conversely, the *hla* repression was described to result from Rot action through *agr*-independent pathway by *saeRS* repression and by *sarT* (230, 312, 313). Additional complexity in *hla* expression

regulation can be also provided by variation in the *hla* promoter region. This was already observed to occur in the *hla* promoter region of *S. aureus* isolates of bovine origin (RF122) (200), where SNPs in the promoter (-376, -483, and -484 from the start codon) seem to contribute to a higher binding affinity of the transcription regulator SarZ that result in the hyperproduction of Hla.

The complexity of *hla* regulation makes difficult to interpret differences in *hla* gene expression. In particular, the difference in *hla* expression observed between CA-MRSA and HA-MRSA is not completely understood. Also, how *in vivo* regulation occurs, remains to be defined, since *in vitro* expression do not mirror completely the *in vivo* expression profiles (46, 366, 396).

5. REFERENCES

1. **Adcock, P. M., P. Pastor, F. Medley, J. E. Patterson, and T. V. Murphy.** 1998. Methicillin-resistant *Staphylococcus aureus* in two child care centers. J Infect Dis **178:**577-80.
2. **Aires de Sousa, M., C. Bartzavali, I. Spiliopoulou, I. S. Sanches, M. I. Crisostomo, 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.
3. **Aires de Sousa, M., T. Conceicao, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J Clin Microbiol **43:**5150-7.
4. **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.
5. **Aires de Sousa, M., I. S. Sanches, M. L. Ferro, M. J. Vaz, Z. Saraiva, T. Tendeiro, J. Serra, and H. de Lencastre.** 1998. Intercontinental spread of a multidrug-resistant methicillin-resistant *Staphylococcus aureus* clone. J Clin Microbiol **36:**2590-6.
6. **Aires-de-Sousa, M., T. Conceicao, 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.
7. **Aires-de-Sousa, M., B. Correia, and H. de Lencastre.** 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:**2912-7.
8. **Alekshun, M. N., and S. B. Levy.** 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell **128:**1037-50.
9. **Amorim, M. L., M. Aires de Sousa, I. S. Sanches, R. Sa-Leao, J. M. Cabeda, J. M. Amorim, and H. de Lencastre.** 2002. Clonal and antibiotic resistance profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) from a Portuguese hospital over time. Microb Drug Resist **8:**301-9.
10. **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.
11. **Arede, P., T. Botelho, T. Guevara, I. Uson, D. C. Oliveira, and F. X. Gomis-Ruth.** 2013. Structure-function studies of the staphylococcal methicillin resistance antirepressor MecR2. J Biol Chem **288:**21267-78.
12. **Argudin, M. A., V. Argumosa, M. C. Mendoza, B. Guerra, and M. R. Rodicio.** 2013. Population structure and exotoxin gene content of methicillin-susceptible *Staphylococcus aureus* from Spanish healthy carriers. Microb Pathog **54:**26-33.
13. **Argudin, M. A., M. C. Mendoza, F. J. Mendez, M. C. Martin, 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.
14. **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.
15. **Arvidson, S., and K. Tegmark.** 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int J Med Microbiol **291:**159-70.
16. **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.** 2014. 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.

17. **Baba, T., T. Bae, O. Schneewind, F. Takeuchi, and K. Hiramatsu.** 2008. Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J Bacteriol **190:**300-10.
18. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. 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.
19. **Ballal, A., B. Ray, and A. C. Manna.** 2009. *sarZ*, a *sarA* family gene, is transcriptionally activated by MgrA and is involved in the regulation of genes encoding exoproteins in *Staphylococcus aureus*. J Bacteriol **191:**1656-65.
20. **Barber, M., and M. Rozwadowska-Dowzenko.** 1948. Infection by penicillin-resistant staphylococci. Lancet **2:**641-4.
21. **Bartley, J.** 2002. First case of VRSA identified in Michigan. Infect Control Hosp Epidemiol **23:**480.
22. **Becker, K., B. Ballhausen, R. Kock, 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.
23. **Bekkhoucha, S. N., A. Cady, P. Gautier, F. Itim, and P. Y. Donnio.** 2009. 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.
24. **Bell, J. M., J. D. Turnidge, and A. Sentry.** 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.
25. **Berglund, C., T. Ito, M. Ikeda, X. X. Ma, B. Soderquist, 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:**3512-6.
26. **Berube, B. J., and J. Bubeck Wardenburg.** 2013. *Staphylococcus aureus* alpha-toxin: nearly a century of intrigue. Toxins (Basel) **5:**1140-66.
27. **Bhakdi, S., and J. Tranum-Jensen.** 1991. Alpha-toxin of *Staphylococcus aureus*. Microbiol Rev **55:**733-51.
28. **Blanc, D. S., C. Petignat, A. Wenger, G. Kuhn, Y. Vallet, D. Fracheboud, S. Trachsel, M. Reymond, N. Troillet, H. H. Siegrist, S. Oeuvray, M. Bes, J. Etienne, J. Bille, P. Francioli, and G. Zanetti.** 2007. Changing molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in a small geographic area over an eight-year period. J Clin Microbiol **45:**3729-36.
29. **Bode, L. G., J. A. Kluytmans, H. F. Wertheim, D. Bogaers, C. M. Vandenbroucke-Grauls, R. Roosendaal, A. Troelstra, A. T. Box, A. Voss, I. van der Tweel, A. van Belkum, H. A. Verbrugh, and M. C. Vos.** 2010. Preventing surgical-site infections in nasal carriers of *Staphylococcus aureus*. N Engl J Med **362:**9-17.
30. **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.
31. **Boye, K., M. D. Bartels, I. S. Andersen, J. A. Moller, 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.
32. **Boyle-Vavra, S., and R. S. Daum.** 2007. Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. Lab Invest **87:**3-9.
33. **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.

34. **Breurec, S., C. Fall, R. Pouillot, P. Boisier, S. Brisse, F. Diene-Sarr, S. Djibo, J. Etienne, M. C. Fonkoua, J. D. Perrier-Gros-Claude, C. E. Ramarokoto, F. Randrianirina, J. M. Thiberge, S. B. Zriouil, B. Garin, and F. Laurent.** 2011. Epidemiology of methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of Panton-Valentine leukocidin genes. Clin Microbiol Infect **17:**633-9.
35. **Bronner, S., P. Stoessel, A. Gravet, H. Monteil, and G. Prevost.** 2000. Variable expressions of *Staphylococcus aureus* bicomponent leucotoxins semiquantified by competitive reverse transcription-PCR. Appl Environ Microbiol **66:**3931-8.
36. **Bubeck Wardenburg, J., T. Bae, M. Otto, F. R. Deleo, and O. Schneewind.** 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat Med **13:**1405-6.
37. **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.
38. **Campanile, F., Bongiorno, D., Borbone, S., and S. Stefani.** 2010. Methicillin-resistant *Staphylococcus aureus* Evolution – The Multiple Facets of an Old Pathogen. European Infectious Disease **4(1)**.
39. **CDC.** 1999. Centers for Disease, Control Prevention. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus* - Minnesota and North Dakota, 1997-1999. MMWR Morb Mortal Wkly Rep **48:**707-10.
40. **Cespedes, C., B. Said-Salim, M. Miller, S. H. Lo, B. N. Kreiswirth, R. J. Gordon, P. Vavagiakis, R. S. Klein, and F. D. Lowy.** 2005. The clonality of *Staphylococcus aureus* nasal carriage. J Infect Dis **191:**444-52.
41. **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**:**226-228.
42. **Chambers, H. F., and F. R. Deleo.** 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol **7:**629-41.
43. **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.
44. **Chen, F. J., T. L. Lauderdale, I. W. Huang, H. J. Lo, J. F. Lai, H. Y. Wang, Y. R. Shiau, P. C. Chen, T. Ito, and K. Hiramatsu.** 2005. Methicillin-resistant *Staphylococcus aureus* in Taiwan. Emerg Infect Dis **11:**1760-3.
45. **Chen, F. J., L. K. Siu, J. C. Lin, C. H. Wang, and P. L. Lu.** 2012. Molecular typing and characterization of nasal carriage and community-onset infection methicillin-susceptible *Staphylococcus aureus* isolates in two Taiwan medical centers. BMC Infect Dis **12:**343.
46. **Cheung, A. L., A. S. Bayer, G. Zhang, H. Gresham, and Y. Q. Xiong.** 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. FEMS Immunol Med Microbiol **40:**1-9.
47. **Cheung, A. L., Y. T. Chien, and A. S. Bayer.** 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. Infect Immun **67:**1331-7.
48. **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:**6462-6.
49. **Cheung, A. L., M. R. Yeaman, P. M. Sullam, M. D. Witt, and A. S. Bayer.** 1994. Role of the *sar* locus of *Staphylococcus aureus* in induction of endocarditis in rabbits. Infect Immun **62:**1719-25.
50. **Chini, V., E. Petinaki, A. Foka, S. Paratiras, G. Dimitracopoulos, and I. Spiliopoulou.** 2006. Spread of *Staphylococcus aureus* clinical isolates carrying Panton-Valentine leukocidin genes during a 3-year period in Greece. Clin Microbiol Infect **12:**29-34.
51. **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.

52. **Clarke, S. R., and S. J. Foster.** 2006. Surface adhesins of *Staphylococcus aureus*. Adv Microb Physiol **51:**187-224.
53. **Clatworthy, A. E., E. Pierson, and D. T. Hung.** 2007. Targeting virulence: a new paradigm for antimicrobial therapy. Nat Chem Biol **3:**541-8.
54. **Coelho, C., C. Torres, H. Radhouani, L. Pinto, C. Lozano, E. Gomez-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.
55. **Conceicao, T., M. Aires de Sousa, M. Miragaia, E. Paulino, R. Barroso, M. J. Brito, T. Sardinha, L. Sancho, H. Carreiro, G. de Sousa, C. Machado Mdo, and H. de Lencastre.** 2012. *Staphylococcus aureus* reservoirs and transmission routes in a Portuguese Neonatal Intensive Care Unit: a 30-month surveillance study. Microb Drug Resist **18:**116-24.
56. **Conceicao, T., M. Aires-de-Sousa, M. Fuzi, A. Toth, J. Paszti, E. Ungvari, 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.
57. **Conceicao, T., M. Aires-de-Sousa, N. Pona, M. J. Brito, C. Barradas, R. Coelho, T. Sardinha, L. Sancho, G. de Sousa, C. Machado Mdo, and H. de Lencastre.** 2011. High prevalence of ST121 in community-associated methicillin-susceptible *Staphylococcus aureus* lineages responsible for skin and soft tissue infections in Portuguese children. Eur J Clin Microbiol Infect Dis **30:**293-7.
58. **Conceicao, 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.
59. **Conceicao, T., I. Santos Silva, H. de Lencastre, and M. Aires-de-Sousa.** 2014. *Staphylococcus aureus* nasal carriage among patients and health care workers in Sao Tome and Principe. Microb Drug Resist **20:**57-66.
60. **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.
61. **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.
62. **Cosgrove, S. E., G. Sakoulas, E. N. Perencevich, M. J. Schwaber, A. W. Karchmer, and Y. Carmeli.** 2003. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. Clin Infect Dis **36:**53-9.
63. **Creech, C. B., 2nd, D. S. Kernodle, A. Alsentzer, C. Wilson, and K. M. Edwards.** 2005. Increasing rates of nasal carriage of methicillin-resistant *Staphylococcus aureus* in healthy children. Pediatr Infect Dis J **24:**617-21.
64. **Crisostomo, 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.
65. **Crossley, K. B., and G. L. Archer.** 1997. The staphylococci in human disease, 1st edition. Churchill Livingstone Inc., New York.

66. **Cuevas, O., E. Cercenado, E. Bouza, C. Castellares, P. Trincado, R. Cabrera, A. Vindel, and S. Spanish Group for the Study of.** 2007. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Spain: a multicentre prevalence study (2002). Clin Microbiol Infect **13:**250-6.
67. **Daskalaki, M., J. R. Otero, F. Sanz, and F. Chaves.** 2007. Bacteremia due to clonally derived methicillin-resistant, gentamicin-susceptible isolates and methicillin-susceptible, gentamicin-resistant isolates of *Staphylococcus aureus*. J Clin Microbiol **45:**3446-8.
68. **Daum, R. S., and B. Spellberg.** 2012. Progress toward a *Staphylococcus aureus* vaccine. Clin Infect Dis **54:**560-7.
69. **David, M. Z., S. Boyle-Vavra, D. L. Zychowski, and R. S. Daum.** 2011. Methicillin-susceptible *Staphylococcus aureus* as a predominantly healthcare-associated pathogen: a possible reversal of roles? PLoS One **6:**e18217.
70. **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.
71. **David, M. Z., R. S. Daum, A. S. Bayer, H. F. Chambers, V. G. Fowler, Jr., L. G. Miller, B. Ostrowsky, A. Baesa, S. Boyle-Vavra, S. J. Eells, S. Garcia-Houchins, P. Gialanella, R. Macias-Gil, T. H. Rude, F. Ruffin, J. J. Sieth, J. Volinski, and B. Spellberg.** 2014. *Staphylococcus aureus* Bacteremia at 5 US Academic Medical Centers, 2008-2011: Significant Geographic Variation in Community-Onset Infections. Clin Infect Dis **59:**798-807.
72. **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; author reply 519-20.
73. **Davis, S. L., M. B. Perri, S. M. Donabedian, C. Manierski, A. Singh, D. Vager, N. Z. Haque, K. Speirs, R. R. Muder, B. Robinson-Dunn, M. K. Hayden, and M. J. Zervos.** 2007. Epidemiology and outcomes of community-associated methicillin-resistant *Staphylococcus aureus* infection. J Clin Microbiol **45:**1705-11.
74. **de Kraker, M. E., P. G. Davey, H. Grundmann, and B. s. group.** 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Med **8:**e1001104.
75. **de Lencastre, H., M. Chung, and H. Westh.** 2000. Archaic strains of methicillin-resistant *Staphylococcus aureus*: molecular and microbiological properties of isolates from the 1960s in Denmark. Microb Drug Resist **6:**1-10.
76. **de Lencastre, H., D. Oliveira, and A. Tomasz.** 2007. Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. Curr Opin Microbiol **10:**428-35.
77. **Del Giudice, P., V. Blanc, F. Durupt, M. Bes, J. P. Martinez, E. Counillon, G. Lina, F. Vandenesch, and J. Etienne.** 2006. Emergence of two populations of methicillin-resistant *Staphylococcus aureus* with distinct epidemiological, clinical and biological features, isolated from patients with community-acquired skin infections. Br J Dermatol **154:**118-24.
78. **DeLeo, F. R., A. D. Kennedy, L. Chen, J. Bubeck Wardenburg, S. D. Kobayashi, B. Mathema, K. R. Braughton, A. R. Whitney, A. E. Villaruz, C. A. Martens, S. F. Porcella, M. J. McGavin, M. Otto, J. M. Musser, and B. N. Kreiswirth.** 2011. Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus*. Proc Natl Acad Sci U S A **108:**18091-6.
79. **DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers.** 2010. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet **375:**1557-68.
80. **den Heijer, C. D. J., E. M. E. 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. The Lancet Infectious Diseases **13:**409-415.
81. **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.

82. **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.
83. **Deurenberg, R. H., P. S. Beisser, M. J. Visschers, C. Driessen, and E. E. Stobberingh.** 2010. Molecular typing of methicillin-susceptible *Staphylococcus aureus* isolates collected in the Yogyakarta area in Indonesia, 2006. Clin Microbiol Infect **16:**92-4.
84. **Deurenberg, R. H., and E. E. Stobberingh.** 2008. The evolution of *Staphylococcus aureus*. Infect Genet Evol **8:**747-63.
85. **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.
86. **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.
87. **DGS.** 2013. Direção-Geral da Saúde, Departamento da Qualidade na Saúde. Portugal - Controlo da Infeção e Resistência aos Antimicrobianos em números - 2013. Portugal:DGS. www.dgs.pt [On line].
88. **DGS.** 2012. Direção-Geral da Saúde, Departamento da Qualidade na Saúde. Prevalência de Infeção Adquirida no Hospital e do Uso de Antimicrobianos nos Hospitais Portugueses, Inquérito 2012. Portugal:DGS. www.dgs.pt [On line].
89. **DH.** Department of Health. London. [Accessed September 2014]. http://www.dh.gov.uk [On line].
90. **Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, M. Beach, and S. P. Group.** 2001. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis **32 Suppl 2:**S114-32.
91. **Diep, B. A., H. F. Chambers, C. J. Graber, J. D. Szumowski, L. G. Miller, L. L. Han, J. H. Chen, F. Lin, J. Lin, T. H. Phan, H. A. Carleton, L. K. McDougal, F. C. Tenover, D. E. Cohen, K. H. Mayer, G. F. Sensabaugh, and F. Perdreau-Remington.** 2008. Emergence of multidrug-resistant, community-associated, methicillin-resistant *Staphylococcus aureus* clone USA300 in men who have sex with men. Ann Intern Med **148:**249-57.
92. **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.
93. **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.
94. **Dohin, B., Y. Gillet, R. Kohler, G. Lina, F. Vandenesch, P. Vanhems, D. Floret, and J. Etienne.** 2007. Pediatric bone and joint infections caused by Panton-Valentine leukocidin-positive *Staphylococcus aureus*. Pediatr Infect Dis J **26:**1042-8.
95. **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.
96. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J Bacteriol **183:**7341-53.

97. **ECDC.** 2012. European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2012. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm. http://www.ecdc.europa.eu/. [Online].
98. **ECDC.** 2013. European Centre for Disease Prevention and Control. Point prevalence survey of healthcare associated infections and antimicrobial use in European acute care hospitals. Stockholm. July 2013 http://www.ecdc.europa.eu/. [Online].
99. **ECDC.** 2010. European Centre for Disease Prevention and Control. Surveillance of antimicrobial consumption in Europe, 2010. Stockholm, March 2013. http://www.ecdc.europa.eu/ [Online].
100. **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.
101. **Ellis, M. W., D. R. Hospenthal, D. P. Dooley, P. J. Gray, and C. K. Murray.** 2004. Natural history of community-acquired methicillin-resistant *Staphylococcus aureus* colonization and infection in soldiers. Clin Infect Dis **39:**971-9.
102. **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.
103. **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.
104. **Espadinha, D., N. A. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, and H. de Lencastre.** 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.
105. **Fang, H., G. Hedin, G. Li, and C. E. Nord.** 2008. Genetic diversity of community-associated methicillin-resistant *Staphylococcus aureus* in southern Stockholm, 2000-2005. Clin Microbiol Infect **14:**370-6.
106. **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.
107. **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.** 2003. How clonal is *Staphylococcus aureus*? J Bacteriol **185:**3307-16.
108. **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.
109. **Fleming, A.** 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *Bacillus infleunzae*. British]ournal of Experimental Pathology **10:**226-236.
110. **Foucault, M. L., P. Courvalin, and C. Grillot-Courvalin.** 2009. Fitness cost of VanA-type vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **53:**2354-9.
111. **Francisco, A. P., M. Bugalho, M. Ramirez, and J. A. Carrico.** 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics **10:**152.
112. **Frazee, B. W., J. Lynn, E. D. Charlebois, L. Lambert, D. Lowery, and F. Perdreau-Remington.** 2005. High prevalence of methicillin-resistant *Staphylococcus aureus* in emergency department skin and soft tissue infections. Ann Emerg Med **45:**311-20.
113. **Freire-Moran, L., B. Aronsson, C. Manz, I. C. Gyssens, A. D. So, D. L. Monnet, O. Cars, and E.-E. W. Group.** 2011. Critical shortage of new antibiotics in development against multidrug-resistant bacteria-Time to react is now. Drug Resist Updat **14:**118-24.

114. **Frenay, 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.
115. **French, G. L., J. A. Otter, K. P. Shannon, N. M. Adams, D. Watling, and M. J. Parks.** 2004. Tackling contamination of the hospital environment by methicillin-resistant *Staphylococcus aureus* (MRSA): a comparison between conventional terminal cleaning and hydrogen peroxide vapour decontamination. J Hosp Infect **57:**31-7.
116. **Friedrich, A. W., W. Witte, H. de Lencastre, W. Hryniewicz, J. Scheres, H. Westh, and p. SeqNet.org.** 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**.
117. **Fritz, S. A., J. Garbutt, A. Elward, W. Shannon, and G. A. Storch.** 2008. Prevalence of and risk factors for community-acquired methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* colonization in children seen in a practice-based research network. Pediatrics **121:**1090-8.
118. **Gal, Z., P. Kovacs, F. Hernadi, G. Barabas, L. Kiss, A. Igloi, and I. Szabo.** 2001. Investigation of oxacillin-hydrolyzing beta-lactamase in borderline methicillin-resistant clinical isolates of *Staphylococcus aureus*. Chemotherapy **47:**233-8.
119. **Garcia-Alvarez, 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.
120. **Gardete, S., M. Aires-De-Sousa, A. Faustino, A. M. Ludovice, and H. de Lencastre.** 2008. Identification of the first vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) isolate from a hospital in Portugal. Microb Drug Resist **14:**1-6.
121. **Gardete, S., and A. Tomasz.** 2014. Mechanisms of vancomycin resistance in *Staphylococcus aureus*. J Clin Invest **124:**2836-40.
122. **Garner, J. S., W. R. Jarvis, T. G. Emori, T. C. Horan, and J. M. Hughes.** 1988. CDC definitions for nosocomial infections, 1988. Am J Infect Control **16:**128-40.
123. **Ghebremedhin, B., F. Layer, W. Konig, and B. Konig.** 2008. Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, *16S rRNA*, *hsp60*, *rpoB*, *sodA*, and *tuf* gene sequences. J Clin Microbiol **46:**1019-25.
124. **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, 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.
125. **Giraudo, A. T., A. Calzolari, A. A. Cataldi, C. Bogni, and R. Nagel.** 1999. The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Microbiol Lett **177:**15-22.
126. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch Microbiol **168:**53-8.
127. **Goering, R. V., R. M. Shawar, N. E. Scangarella, F. P. O'Hara, H. Amrine-Madsen, J. M. West, M. Dalessandro, J. A. Becker, S. L. Walsh, L. A. Miller, S. F. van Horn, E. S. Thomas, and M. E. Twynholm.** 2008. Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials. J Clin Microbiol **46:**2842-7.
128. **Goerke, C., U. Fluckiger, A. Steinhuber, W. Zimmerli, and C. Wolz.** 2001. Impact of the regulatory loci *agr*, *sarA* and *sae* of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis. Mol Microbiol **40:**1439-47.
129. **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.

130. **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.
131. **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. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America **29:**797-800.
132. **Gorwitz, R. J., D. Kruszon-Moran, S. K. McAllister, G. McQuillan, L. K. McDougal, G. E. Fosheim, B. J. Jensen, G. Killgore, F. C. Tenover, and M. J. Kuehnert.** 2008. Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001-2004. J Infect Dis **197:**1226-34.
133. **Gowrishankar, S., R. Thenmozhi, K. Balaji, and S. K. Pandian.** 2013. Emergence of methicillin-resistant, vancomycin-intermediate *Staphylococcus aureus* among patients associated with group A Streptococcal pharyngitis infection in southern India. Infect Genet Evol **14:**383-9.
134. **Gray, G. S., and M. Kehoe.** 1984. Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* wood 46. Infect Immun **46:**615-8.
135. **Gray, R. R., A. J. Tatem, J. A. Johnson, A. V. Alekseyenko, O. G. Pybus, M. A. Suchard, and M. Salemi.** 2011. Testing spatiotemporal hypothesis of bacterial evolution using methicillin-resistant *Staphylococcus aureus* ST239 genome-wide data within a bayesian framework. Mol Biol Evol **28:**1593-603.
136. **Grindstaff, T. L., S. A. Saliba, D. J. Mistry, and J. M. Macknight.** 2007. Community-associated methicillin-resistant *Staphylococcus aureus*. N Am J Sports Phys Ther **2:**138-46.
137. **Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med **7:**e1000215.
138. **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.
139. **Grundmann, H., S. Barwolff, A. Tami, M. Behnke, F. Schwab, C. Geffers, E. Halle, U. B. Gobel, R. Schiller, D. Jonas, I. Klare, K. Weist, W. Witte, K. Beck-Beilecke, M. Schumacher, H. Ruden, and P. Gastmeier.** 2005. How many infections are caused by patient-to-patient transmission in intensive care units? Crit Care Med **33:**946-51.
140. **Grundmann, H., A. Tami, S. Hori, M. Halwani, and R. Slack.** 2002. Nottingham *Staphylococcus aureus* population study: prevalence of MRSA among elderly people in the community. BMJ **324:**1365-6.
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. **Hageman, J. C., T. M. Uyeki, J. S. Francis, D. B. Jernigan, J. G. Wheeler, C. B. Bridges, S. J. Barenkamp, D. M. Sievert, A. Srinivasan, M. C. Doherty, L. K. McDougal, G. E. Killgore, U. A. Lopatin, R. Coffman, J. K. MacDonald, S. K. McAllister, G. E. Fosheim, J. B. Patel, and L. C. McDonald.** 2006. Severe community-acquired pneumonia due to *Staphylococcus aureus*, 2003-04 influenza season. Emerg Infect Dis **12:**894-9.
143. **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.
144. **Hanssen, A. M., and J. U. Ericson Sollid.** 2006. SCC*mec* in staphylococci: genes on the move. FEMS Immunol Med Microbiol **46:**8-20.

145. **Hanssen, A. M., A. Fossum, J. Mikalsen, D. S. Halvorsen, G. Bukholm, and J. U. Sollid.** 2005. Dissemination of community-acquired methicillin-resistant *Staphylococcus aureus* clones in northern Norway: sequence types 8 and 80 predominate. J Clin Microbiol **43:**2118-24.
146. **Harris, S. R., E. J. Feil, M. T. Holden, M. A. Quail, E. K. Nickerson, N. Chantratita, S. Gardete, A. Tavares, N. Day, J. A. Lindsay, J. D. Edgeworth, H. de Lencastre, J. Parkhill, S. J. Peacock, and S. D. Bentley.** Evolution of MRSA during hospital transmission and intercontinental spread. Science **327:**469-74.
147. **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.
148. **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.
149. **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.
150. **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.
151. **Hisata, K., K. Kuwahara-Arai, M. Yamanoto, T. Ito, Y. Nakatomi, L. Cui, T. Baba, M. Terasawa, C. Sotozono, S. Kinoshita, Y. Yamashiro, and K. Hiramatsu.** 2005. Dissemination of methicillin-resistant staphylococci among healthy Japanese children. J Clin Microbiol **43:**3364-72.
152. **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.
153. **Hoeger, P. H., W. Lenz, A. Boutonnier, and J. M. Fournier.** 1992. Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. J Infect Dis **165:**1064-8.
154. **Horan, T. C., M. Andrus, and M. A. Dudeck.** 2008. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control **36:**309-32.
155. **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.
156. **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.
157. **Horvath, A., O. Dobay, S. Kardos, A. Ghidan, A. Toth, J. Paszti, E. Ungvari, P. Horvath, K. Nagy, S. Zissman, and M. Fuzi.** 2012. Varying fitness cost associated with resistance to fluoroquinolones governs clonal dynamic of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis **31:**2029-36.
158. **HPA.** 2009. Health Protection Agency. Healthcare-associated Infections in England: 2008-2009 Report. London:HPA. Sept 2009. http://www.hpa.org.uk/web/HPAwebFile/HPAwebC/1252326222452 [Online].
159. **Hsu, L. Y., T. H. Koh, K. Singh, M. L. Kang, A. Kurup, and B. H. Tan.** 2005. Dissemination of multisusceptible methicillin-resistant *Staphylococcus aureus* in Singapore. J Clin Microbiol **43:**2923-5.
160. **Huang, Y. C., and C. J. Chen.** 2011. Community-associated meticillin-resistant *Staphylococcus aureus* in children in Taiwan, 2000s. Int J Antimicrob Agents **38:**2-8.
161. **Huntzinger, E., S. Boisset, C. Saveanu, Y. Benito, T. Geissmann, A. Namane, G. Lina, J. Etienne, B. Ehresmann, C. Ehresmann, A. Jacquier, F. Vandenesch, and P. Romby.** 2005.

Staphylococcus aureus RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. EMBO J **24:**824-35.

162. **Hussain, F. M., S. Boyle-Vavra, and R. S. Daum.** 2001. Community-acquired methicillin-resistant *Staphylococcus aureus* colonization in healthy children attending an outpatient pediatric clinic. Pediatr Infect Dis J **20:**763-7.
163. **International Working Group on the Classification of Staphylococcal Cassette Chromosome, E.** 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother **53:**4961-7.
164. **Ito, T., M. Iijima, T. Fukushima, M. Nonoyama, M. Ishii, T. Baranovich, T. Otsuka, T. Takano, and T. Yamamoto.** 2008. Pediatric pneumonia death caused by community-acquired methicillin-resistant *Staphylococcus aureus*, Japan. Emerg Infect Dis **14:**1312-4.
165. **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.
166. **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.
167. **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.
168. **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.
169. **Jenkins, T. C., B. D. McCollister, R. Sharma, K. K. McFann, N. E. Madinger, M. Barron, M. Bessesen, C. S. Price, and W. J. Burman.** 2009. Epidemiology of healthcare-associated bloodstream infection caused by USA300 strains of methicillin-resistant *Staphylococcus aureus* in 3 affiliated hospitals. Infect Control Hosp Epidemiol **30:**233-41.
170. **Jevons, M. P.** 1961. "Celbenin"-resistant staphylococci. British Medical Journal **1:**124.
171. **Johnson, A. P., H. M. Aucken, S. Cavendish, M. Ganner, M. C. Wale, M. Warner, D. M. Livermore, and B. D. Cookson.** 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.
172. **Johnson, A. P., J. Davies, R. Guy, J. Abernethy, E. Sheridan, A. Pearson, and G. Duckworth.** 2012. Mandatory surveillance of methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia in England: the first 10 years. J Antimicrob Chemother **67:**802-9.
173. **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.
174. **Kampf, G., and A. Kramer.** 2004. Epidemiologic background of hand hygiene and evaluation of the most important agents for scrubs and rubs. Clin Microbiol Rev **17:**863-93, table of contents.
175. **Kaplan, S. L., K. G. Hulten, B. E. Gonzalez, W. A. Hammerman, L. Lamberth, J. Versalovic, and E. O. Mason, Jr.** 2005. Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. Clin Infect Dis **40:**1785-91.
176. **Karden-Lilja, M., S. Ibrahem, J. Vuopio-Varkila, S. Salmenlinna, O. Lyytikainen, L. Siira, and A. Virolainen.** 2007. Panton-Valentine leukocidin genes and staphylococcal chromosomal cassette *mec* types amongst Finnish community-acquired methicillin-resistant *Staphylococcus aureus* strains, 1997-1999. Eur J Clin Microbiol Infect Dis **26:**729-33.
177. **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.

178. **Kehoe, M., J. Duncan, T. Foster, N. Fairweather, and G. Dougan.** 1983. Cloning, expression, and mapping of the *Staphylococcus aureus* alpha-hemolysin determinant in *Escherichia coli* K-12. Infect Immun **41:**1105-11.
179. **Khokhlova, O., Y. Tomita, W. C. Hung, T. Takano, Y. Iwao, W. Higuchi, A. Nishiyama, I. Reva, and T. Yamamoto.** 2012. 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.
180. **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.
181. **King, M. D., B. J. Humphrey, Y. F. Wang, E. V. Kourbatova, S. M. Ray, and H. M. Blumberg.** 2006. Emergence of community-acquired methicillin-resistant *Staphylococcus aureus* USA 300 clone as the predominant cause of skin and soft-tissue infections. Ann Intern Med **144:**309-17.
182. **Kirby, W. M.** 1944. Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant Staphylococci. Science **99:**452-3.
183. **Kirmani, N., C. U. Tuazon, H. W. Murray, A. E. Parrish, and J. N. Sheagren.** 1978. *Staphylococcus aureus* carriage rate of patients receiving long-term hemodialysis. Arch Intern Med **138:**1657-9.
184. **Kloos, W. E.** 1998. *Staphylococcus*, pp. 577-632. In: Collier, L., Balows, A., Sussman, M., (eds), Topley and Wilson's Microbiology and Microbial Infections. Volume 2: Systematic Bacteriology. 9th Ed. Edward Arnold, London.
185. **Kluytmans, J., A. van Belkum, and H. Verbrugh.** 1997. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev **10:**505-20.
186. **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.
187. **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.
188. **Koser, C. U., M. T. Holden, M. J. Ellington, E. J. Cartwright, N. M. Brown, A. L. Ogilvy-Stuart, L. Y. Hsu, C. Chewapreecha, N. J. Croucher, S. R. Harris, M. Sanders, M. C. Enright, G. Dougan, S. D. Bentley, J. Parkhill, L. J. Fraser, J. R. Betley, O. B. Schulz-Trieglaff, G. P. Smith, and S. J. Peacock.** 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med **366:**2267-75.
189. **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.
190. **Krziwanek, K., C. Luger, B. Sammer, S. Stumvoll, M. Stammler, U. Sagel, W. Witte, and H. Mittermayer.** 2008. MRSA in Austria--an overview. Clin Microbiol Infect **14:**250-9.
191. **Kuehnert, M. J., D. Kruszon-Moran, H. A. Hill, G. McQuillan, S. K. McAllister, G. Fosheim, L. K. McDougal, J. Chaitram, B. Jensen, S. K. Fridkin, G. Killgore, and F. C. Tenover.** 2006. Prevalence of *Staphylococcus aureus* nasal colonization in the United States, 2001-2002. J Infect Dis **193:**172-9.
192. **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.
193. **Labandeira-Rey, M., F. Couzon, S. Boisset, E. L. Brown, M. Bes, Y. Benito, E. M. Barbu, V. Vazquez, M. Hook, J. Etienne, F. Vandenesch, and M. G. Bowden.** 2007. *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. Science **315:**1130-3.

194. **Lamers, R. P., J. W. Stinnett, G. Muthukrishnan, C. L. Parkinson, and A. M. Cole.** 2011. Evolutionary analyses of *Staphylococcus aureus* identify genetic relationships between nasal carriage and clinical isolates. PLoS One **6:**e16426.
195. **Lannergard, J., T. Norstrom, and D. Hughes.** 2009. Genetic determinants of resistance to fusidic acid among clinical bacteremia isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother **53:**2059-65.
196. **Larsen, A. R., S. Bocher, M. Stegger, R. Goering, L. V. Pallesen, and R. Skov.** 2008. Epidemiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. J Clin Microbiol **46:**62-8.
197. **Len, K. A., L. Bergert, S. Patel, M. Melish, C. Kimata, and G. Erdem.** 2010. Community-acquired *Staphylococcus aureus* pneumonia among hospitalized children in Hawaii. Pediatr Pulmonol **45:**898-905.
198. **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.
199. **Li, M., X. Du, A. E. Villaruz, B. A. Diep, D. Wang, Y. Song, Y. Tian, J. Hu, F. Yu, Y. Lu, and M. Otto.** 2012. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. Nat Med **18:**816-9.
200. **Liang, X., J. W. Hall, J. Yang, M. Yan, K. Doll, R. Bey, and Y. Ji.** 2011. Identification of single nucleotide polymorphisms associated with hyperproduction of alpha-toxin in *Staphylococcus aureus*. PLoS One **6:**e18428.
201. **Liang, X., L. Zheng, C. Landwehr, D. Lunsford, D. Holmes, and Y. Ji.** 2005. Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. J Bacteriol **187:**5486-92.
202. **Lina, G., Y. Piemont, 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.
203. **Linde, H., F. Wagenlehner, B. Strommenger, I. Drubel, J. Tanzer, U. Reischl, U. Raab, C. Holler, K. G. Naber, W. Witte, F. Hanses, B. Salzberger, and N. Lehn.** 2005. Healthcare-associated outbreaks and community-acquired infections due to MRSA carrying the Panton-Valentine leucocidin gene in southeastern Germany. Eur J Clin Microbiol Infect Dis **24:**419-22.
204. **Lindsay, J. A.** 2010. Genomic variation and evolution of *Staphylococcus aureus*. Int J Med Microbiol **300:**98-103.
205. **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.
206. **Lipsky, B. A., R. E. Pecoraro, M. S. Chen, and T. D. Koepsell.** 1987. Factors affecting staphylococcal colonization among NIDDM outpatients. Diabetes Care **10:**483-6.
207. **Liu, C., C. J. Graber, M. Karr, B. A. Diep, L. Basuino, B. S. Schwartz, M. C. Enright, S. J. O'Hanlon, J. C. Thomas, F. Perdreau-Remington, S. Gordon, H. Gunthorpe, R. Jacobs, P. Jensen, G. Leoung, J. S. Rumack, and H. F. Chambers.** 2008. A population-based study of the incidence and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* disease in San Francisco, 2004-2005. Clin Infect Dis **46:**1637-46.
208. **Lo, W. T., C. C. Wang, W. J. Lin, S. R. Wang, C. S. Teng, C. F. Huang, and S. J. Chen.** 2010. Changes in the nasal colonization with methicillin-resistant *Staphylococcus aureus* in children: 2004-2009. PLoS One **5:**e15791.
209. **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.
210. **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.

211. **Lowy, F. D.** 2011. How *Staphylococcus aureus* adapts to its host. N Engl J Med **364:**1987-90.
212. **Lowy, F. D.** 1998. *Staphylococcus aureus* infections. N Engl J Med **339:**520-32.
213. **Lowy, F. D., A. E. Aiello, M. Bhat, V. D. Johnson-Lawrence, M. H. Lee, E. Burrell, L. N. Wright, G. Vasquez, and E. L. Larson.** 2007. *Staphylococcus aureus* colonization and infection in New York State prisons. J Infect Dis **196:**911-8.
214. **Luong, T. T., S. W. Newell, and C. Y. Lee.** 2003. Mgr, a novel global regulator in *Staphylococcus aureus*. J Bacteriol **185:**3703-10.
215. **Ma, X. X., 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.
216. **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.
217. **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.
218. **Maier, J., H. Melzl, U. Reischl, I. Drubel, W. Witte, N. Lehn, and H. Linde.** 2005. Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* in Germany associated with travel or foreign family origin. Eur J Clin Microbiol Infect Dis **24:**637-9.
219. **Marchese, A., L. Gualco, E. Maioli, and E. Debbia.** 2009. Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int J Antimicrob Agents **34:**424-8.
220. **Maree, C. L., S. J. Eells, J. Tan, E. A. Bancroft, M. Malek, N. T. Harawa, M. J. Lewis, E. Santana, and L. G. Miller.** 2010. Risk factors for infection and colonization with community-associated methicillin-resistant *Staphylococcus aureus* in the Los Angeles County jail: a case-control study. Clin Infect Dis **51:**1248-57.
221. **Marshall, C., and D. Spelman.** 2007. Re: 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.
222. **Martinez-Aguilar, G., A. Avalos-Mishaan, K. Hulten, W. Hammerman, E. O. Mason, Jr., and S. L. Kaplan.** 2004. Community-acquired, methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* musculoskeletal infections in children. Pediatr Infect Dis J **23:**701-6.
223. **McAdam, P. R., A. Holmes, K. E. Templeton, and J. R. Fitzgerald.** 2011. Adaptive evolution of *Staphylococcus aureus* during chronic endobronchial infection of a cystic fibrosis patient. PLoS One **6:**e24301.
224. **McAdam, P. R., K. E. Templeton, G. F. Edwards, M. T. Holden, E. J. Feil, D. M. Aanensen, H. J. Bargawi, B. G. Spratt, S. D. Bentley, J. Parkhill, M. C. Enright, A. Holmes, E. K. Girvan, P. A. Godfrey, M. Feldgarden, A. M. Kearns, A. Rambaut, D. A. Robinson, and J. R. Fitzgerald.** 2012. Molecular tracing of the emergence, adaptation, and transmission of hospital-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A **109:**9107-12.
225. **McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster.** 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. Mol Microbiol **11:**237-48.
226. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 2003. 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.
227. **McDougal, L. K., and C. Thornsberry.** 1986. The role of beta-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. J Clin Microbiol **23:**832-9.

228. **McGavin, M. J., B. Arsic, and N. N. Nickerson.** 2012. Evolutionary blueprint for host- and niche-adaptation in *Staphylococcus aureus* clonal complex CC30. Front Cell Infect Microbiol **2:**48.
229. **McHugh, C. G., and L. W. Riley.** 2004. Risk factors and costs associated with methicillin-resistant *Staphylococcus aureus* bloodstream infections. Infect Control Hosp Epidemiol **25:**425-30.
230. **McNamara, P. J., and A. S. Bayer.** 2005. A *rot* mutation restores parental virulence to an *agr*-null *Staphylococcus aureus* strain in a rabbit model of endocarditis. Infect Immun **73:**3806-9.
231. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J Bacteriol **182:**3197-203.
232. **Melles, D. C., W. B. van Leeuwen, H. A. Boelens, J. K. Peeters, H. A. Verbrugh, and A. van Belkum.** 2006. Panton-Valentine leukocidin genes in *Staphylococcus aureus*. Emerg Infect Dis **12:**1174-5.
233. **Melo-Cristino, J., C. Resina, V. Manuel, L. Lito, and M. Ramirez.** 2013. First case of infection with vancomycin-resistant *Staphylococcus aureus* in Europe. Lancet **382:**205.
234. **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.
235. **Merckoll, P., T. O. 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. **Miko, B. A., C. A. Hafer, C. J. Lee, S. B. Sullivan, M. A. Hackel, B. M. Johnson, S. Whittier, P. Della-Latta, A. C. Uhlemann, and F. D. Lowy.** 2013. Molecular characterization of methicillin-susceptible *Staphylococcus aureus* clinical isolates in the United States, 2004 to 2010. J Clin Microbiol **51:**874-9.
237. **Milheirico, 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.
238. **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.
239. **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.
240. **Miller, L. G., F. Perdreau-Remington, A. S. Bayer, B. Diep, N. Tan, K. Bharadwa, J. Tsui, J. Perlroth, A. Shay, G. Tagudar, U. Ibebuogu, and B. Spellberg.** 2007. Clinical and epidemiologic characteristics cannot distinguish community-associated methicillin-resistant *Staphylococcus aureus* infection from methicillin-susceptible *S. aureus* infection: a prospective investigation. Clin Infect Dis **44:**471-82.
241. **Miller, L. G., F. Perdreau-Remington, G. Rieg, S. Mehdi, J. Perlroth, A. S. Bayer, A. W. Tang, T. O. Phung, and B. Spellberg.** 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N Engl J Med **352:**1445-53.
242. **Miller, M. B., D. J. Weber, J. S. Goodrich, E. B. Popowitch, M. D. Poe, V. Nyugen, T. R. Shope, D. T. Foster, J. R. Miller, and J. Kotch.** 2011. Prevalence and risk factor analysis for methicillin-resistant *Staphylococcus aureus* nasal colonization in children attending child care centers. J Clin Microbiol **49:**1041-7.
243. **Moet, G. J., R. N. Jones, D. J. Biedenbach, M. G. Stilwell, and T. R. Fritsche.** 2007. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998-2004). Diagn Microbiol Infect Dis **57:**7-13.

244. **Mongkolrattanothai, K., J. C. Aldag, P. Mankin, and B. M. Gray.** 2009. Epidemiology of community-onset *Staphylococcus aureus* infections in pediatric patients: an experience at a Children's Hospital in central Illinois. BMC Infect Dis **9:**112.
245. **Monti, G., P. Tonetto, M. Mostert, and R. Oggero.** 1996. *Staphylococcus aureus* skin colonization in infants with atopic dermatitis. Dermatology **193:**83-7.
246. **Montoya, M., and E. Gouaux.** 2003. Beta-barrel membrane protein folding and structure viewed through the lens of alpha-hemolysin. Biochim Biophys Acta **1609:**19-27.
247. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, V. Albrecht, B. Limbago, D. A. Talan, and E. M. I. N. S. Group.** 2012. Prevalence of methicillin-resistant *Staphylococcus aureus* as an etiology of community-acquired pneumonia. Clin Infect Dis **54:**1126-33.
248. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **355:**666-74.
249. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, D. A. Talan, and E. M. I. N. S. Group.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **355:**666-74.
250. **Moravvej, Z., F. Estaji, E. Askari, K. Solhjou, M. Naderi Nasab, and S. Saadat.** 2013. Update on the global number of vancomycin-resistant *Staphylococcus aureus* (VRSA) strains. Int J Antimicrob Agents **42:**370-1.
251. **Morgan, M.** 2008. Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? J Antimicrob Chemother **62:**1181-7.
252. **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.
253. **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.
254. **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.
255. **Nakamura, M. M., K. L. Rohling, M. Shashaty, H. Lu, Y. W. Tang, and K. M. Edwards.** 2002. Prevalence of methicillin-resistant *Staphylococcus aureus* nasal carriage in the community pediatric population. Pediatr Infect Dis J **21:**917-22.
256. **Nazareth, R., J. Goncalves-Pereira, A. Tavares, M. Miragaia, H. de Lencastre, J. Silvestre, P. Freitas, E. Goncalves, F. Martins, V. Mendes, C. Tapadinhas, and P. Povoa.** 2012. Community-associated methicillin-resistant *Staphylococcus aureus* infection in Portugal. Rev Port Pneumol **18:**34-8.
257. **Nguyen, M. H., C. A. Kauffman, R. P. Goodman, C. Squier, R. D. Arbeit, N. Singh, M. M. Wagener, and V. L. Yu.** 1999. Nasal carriage of and infection with *Staphylococcus aureus* in HIV-infected patients. Ann Intern Med **130:**221-5.
258. **Nilsson, P., and T. Ripa.** 2006. *Staphylococcus aureus* throat colonization is more frequent than colonization in the anterior nares. J Clin Microbiol **44:**3334-9.
259. **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.
260. **Noskin, G. A., R. J. Rubin, J. J. Schentag, J. Kluytmans, E. C. Hedblom, C. Jacobson, M. Smulders, E. Gemmen, and M. Bharmal.** 2007. National trends in *Staphylococcus aureus* infection rates: impact on economic burden and mortality over a 6-year period (1998-2003). Clin Infect Dis **45:**1132-40.

261. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429-49.
262. **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.
263. **Nubel, 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.
264. **Nulens, E., E. E. Stobberingh, H. van Dessel, S. Sebastian, F. H. van Tiel, P. S. Beisser, and R. H. Deurenberg.** 2008. Molecular characterization of *Staphylococcus aureus* bloodstream isolates collected in a Dutch University Hospital between 1999 and 2006. J Clin Microbiol **46:**2438-41.
265. **O'Brien, F. G., T. T. Lim, F. N. Chong, G. W. Coombs, M. C. Enright, D. A. Robinson, A. Monk, B. Said-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.
266. **O'Riordan, K., and J. C. Lee.** 2004. *Staphylococcus aureus* capsular polysaccharides. Clin Microbiol Rev **17:**218-34.
267. **Ogston, A.** 1882. Micrococcus Poisoning. J Anat Physiol **17:**24-58.
268. **Okubo, T., S. Yabe, T. Otsuka, Y. Takizawa, T. Takano, S. Dohmae, W. Higuchi, H. Tsukada, F. Gejyo, M. Uchiyama, and T. Yamamoto.** 2008. Multifocal pelvic abscesses and osteomyelitis from community-acquired methicillin-resistant *Staphylococcus aureus* in a 17-year-old basketball player. Diagn Microbiol Infect Dis **60:**313-8.
269. **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.
270. **Oliveira, D. C., I. Crisostomo, I. Santos-Sanches, P. Major, C. R. Alves, M. Aires-de-Sousa, M. K. Thege, and H. de Lencastre.** 2001. Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **39:**574-80.
271. **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.
272. **Oliveira, D. C., C. Milheirico, and H. de Lencastre.** 2006. Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCC*mec* type VI. Antimicrob Agents Chemother **50:**3457-9.
273. **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.
274. **Orscheln, R. C., D. A. Hunstad, S. A. Fritz, J. A. Loughman, K. Mitchell, E. K. Storch, M. Gaudreault, P. L. Sellenriek, J. R. Armstrong, E. R. Mardis, and G. A. Storch.** 2009. contribution of genetically restricted, methicillin-susceptible strains to the ongoing epidemic of community-acquired *Staphylococcus aureus* infections. Clin Infect Dis **49:**536-42.
275. **Otter, J. A., and G. L. French.** 2008. Community-associated meticillin-resistant *Staphylococcus aureus* in injecting drug users and the homeless in south London. J Hosp Infect **69:**198-200.
276. **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.
277. **Otter, J. A., and G. L. French.** 2008. The emergence of community-associated methicillin-resistant *Staphylococcus aureus* at a London teaching hospital, 2000-2006. Clin Microbiol Infect **14:**670-6.
278. **Otter, J. A., S. Yezli, and G. L. French.** 2011. The role played by contaminated surfaces in the transmission of nosocomial pathogens. Infect Control Hosp Epidemiol **32:**687-99.

279. **Otto, M.** 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu Rev Microbiol **64:**143-62.
280. **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.
281. **Pantucek, R., P. Svec, J. J. Dajcs, I. Machova, J. Cernohlavkova, O. Sedo, T. Gelbicova, I. Maslanova, J. Doskar, Z. Zdrahal, V. Ruzickova, and I. Sedlacek.** 2013. *Staphylococcus petrasii* sp. nov. including *S. petrasii* subsp. *petrasii* subsp. nov. and *S. petrasii* subsp. *croceilyticus* subsp. nov., isolated from human clinical specimens and human ear infections. Syst Appl Microbiol **36:**90-5.
282. **Patel, N. C., I. C. Hanson, and L. M. Noroski.** 2008. Methicillin-susceptible *Staphylococcus aureus* brain abscess in common variable immunodeficiency after an 8-month gap in return to the immunologist. J Allergy Clin Immunol **122:**1036-7; author reply 1037.
283. **Paterson, G. K., E. M. Harrison, and M. A. Holmes.** 2014. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. Trends Microbiol **22:**42-7.
284. **Pearson, A., A. Chronias, and M. Murray.** 2009. Voluntary and mandatory surveillance for methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) bacteraemia in England. J Antimicrob Chemother **64 Suppl 1:**i11-7.
285. **Perez-Roth, E., F. Lorenzo-Diaz, N. Batista, A. Moreno, and S. Mendez-Alvarez.** 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.
286. **Perez-Vazquez, M., A. Vindel, C. Marcos, J. Oteo, O. Cuevas, P. Trincado, V. Bautista, H. Grundmann, J. Campos, and E. S. s.-t. 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.
287. **Perichon, B., and P. Courvalin.** 2009. VanA-type vancomycin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **53:**4580-7.
288. **Pomba, C., F. M. Baptista, N. Couto, F. Loucao, and H. Hasman.** 2010. Methicillin-resistant *Staphylococcus aureus* CC398 isolates with indistinguishable ApaI restriction patterns in colonized and infected pigs and humans. J Antimicrob Chemother **65:**2479-81.
289. **Pomba, C., H. Hasman, L. M. Cavaco, J. D. da Fonseca, and F. M. Aarestrup.** 2009. First description of meticillin-resistant *Staphylococcus aureus* (MRSA) CC30 and CC398 from swine in Portugal. Int J Antimicrob Agents **34:**193-4.
290. **Price, L. B., M. Stegger, H. Hasman, M. Aziz, J. Larsen, P. 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**.
291. **Proctor, R. A.** 2012. Is there a future for a *Staphylococcus aureus* vaccine? Vaccine **30:**2921-7.
292. **Randolph, A. G., F. Vaughn, R. Sullivan, L. Rubinson, B. T. Thompson, G. Yoon, E. Smoot, T. W. Rice, L. L. Loftis, M. Helfaer, A. Doctor, M. Paden, H. Flori, C. Babbitt, A. L. Graciano, R. Gedeit, R. C. Sanders, J. S. Giuliano, J. Zimmerman, T. M. Uyeki, I. Pediatric Acute Lung, N. Sepsis Investigator's, L. the National Heart, and A. C. T. N. Blood Institute.** 2011. Critically ill children during the 2009-2010 influenza pandemic in the United States. Pediatrics **128:**e1450-8.
293. **Rasigade, J. P., F. Laurent, G. Lina, H. Meugnier, M. Bes, F. Vandenesch, J. Etienne, and A. Tristan.** 2010. Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981-2007. J Infect Dis **201:**1589-97.
294. **Redziniak, D. E., D. R. Diduch, K. Turman, J. Hart, T. L. Grindstaff, J. M. MacKnight, and D. J. Mistry.** 2009. Methicillin-resistant *Staphylococcus aureus* (MRSA) in the Athlete. Int J Sports Med **30:**557-62.

295. **Reyes, D., D. O. Andrey, A. Monod, W. L. Kelley, G. Zhang, and A. L. Cheung.** 2011. Coordinated regulation by AgrA, SarA, and SarR to control *agr* expression in *Staphylococcus aureus*. J Bacteriol **193:**6020-31.

296. **Rijnders, M. I., R. H. Deurenberg, M. L. Boumans, J. A. Hoogkamp-Korstanje, P. S. Beisser, and E. E. Stobberingh.** 2009. Population structure of *Staphylococcus aureus* strains isolated from intensive care unit patients in the netherlands over an 11-year period (1996 to 2006). J Clin Microbiol **47:**4090-5.

297. **Robert, J., J. Etienne, X. Bertrand, and Onerba.** 2005. Methicillin-resistant *Staphylococcus aureus* producing Panton-Valentine leukocidin in a retrospective case series from 12 French hospital laboratories, 2000-2003. Clin Microbiol Infect **11:**585-7.

298. **Robinson, D. A., and M. C. Enright.** 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **47:**3926-34.

299. **Robinson, D. A., and M. C. Enright.** 2004. Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect **10:**92-7.

300. **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.

301. **Rogasch, K., V. Ruhmling, J. Pane-Farre, D. Hoper, C. Weinberg, S. Fuchs, M. Schmudde, B. M. Broker, C. Wolz, M. Hecker, and S. Engelmann.** 2006. Influence of the two-component system SaeRS on global gene expression in two different *Staphylococcus aureus* strains. J Bacteriol **188:**7742-58.

302. **Rolinson, G. N., F. R. Batchelor, D. Butterworth, J. Cameron-Wood, M. Cole, G. C. Eustace, M. V. Hart, M. Richards, and E. B. Chain.** 1960. Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. Nature **187:**236-7.

303. **Rolo, J., M. Miragaia, A. Turlej-Rogacka, J. Empel, O. Bouchami, N. A. Faria, A. Tavares, W. Hryniewicz, A. C. Fluit, H. de Lencastre, and C. W. Group.** 2012. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One **7:**e34768.

304. **Rosenbach, F.** 1884. Microorganismen bei den Wund-Infections-Krankheiten des Menschen. J.F. Bergmann, Wiesbaden, pp. 1-122.

305. **Rossi, F., L. Diaz, A. Wollam, D. Panesso, Y. Zhou, S. Rincon, A. Narechania, G. Xing, T. S. Di Gioia, A. Doi, T. T. Tran, J. Reyes, J. M. Munita, L. P. Carvajal, A. Hernandez-Roldan, D. Brandao, I. M. van der Heijden, B. E. Murray, P. J. Planet, G. M. Weinstock, and C. A. Arias.** 2014. Transferable vancomycin resistance in a community-associated MRSA lineage. N Engl J Med **370:**1524-31.

306. **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.

307. **Roundtree, P. M., and M. A. Beard.** 1958. Further observations on infections with phage type 80 staphylococci in Australia. Med J Aust **2:**789–795.

308. **Rubinstein, E., M. H. Kollef, and D. Nathwani.** 2008. Pneumonia caused by methicillin-resistant *Staphylococcus aureus*. Clin Infect Dis **46 Suppl 5:**S378-85.

309. **Ruimy, R., A. Maiga, L. Armand-Lefevre, I. Maiga, A. Diallo, A. K. Koumare, K. Ouattara, S. Soumare, K. Gaillard, J. C. Lucet, A. Andremont, and E. J. Feil.** 2008. The carriage population of *Staphylococcus aureus* from Mali is composed of a combination of pandemic clones and the divergent Panton-Valentine leukocidin-positive genotype ST152. J Bacteriol **190:**3962-8.

310. **Sa-Leao, R., I. S. Sanches, I. Couto, C. R. Alves, and H. de Lencastre.** 2001. Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist **7:**237-45.

311. **Sa-Leao, R., I. Santos Sanches, D. Dias, I. Peres, R. M. Barros, and H. de Lencastre.** 1999. Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in international samples: relics of a formerly widely disseminated strain? J Clin Microbiol **37:**1913-20.
312. **Said-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.
313. **Schmidt, K. A., A. C. Manna, S. Gill, and A. L. Cheung.** 2001. SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. Infect Immun **69:**4749-58.
314. **Sendi, P., and R. A. Proctor.** 2009. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. Trends Microbiol **17:**54-8.
315. **Sexton, T., P. Clarke, E. O'Neill, T. Dillane, and H. Humphreys.** 2006. Environmental reservoirs of methicillin-resistant *Staphylococcus aureus* in isolation rooms: correlation with patient isolates and implications for hospital hygiene. J Hosp Infect **62:**187-94.
316. **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.
317. **Shanson, D. C.** 1992. Antibiotic resistance in *Staphylococcus aureus*, p. 11-20. In M. T. Cafferkey (ed.), Methicillin-resistant *Staphylococcus aureus*: clinical management and laboratory aspects, 1st edition. Informa Health Care, New York.
318. **Shittu, A. O., K. Okon, S. Adesida, O. Oyedara, W. Witte, B. Strommenger, F. Layer, and U. Nubel.** 2011. Antibiotic resistance and molecular epidemiology of *Staphylococcus aureus* in Nigeria. BMC Microbiol **11:**92.
319. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, 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.
320. **Shore, A. C., E. C. Deasy, P. Slickers, G. Brennan, B. O'Connell, S. Monecke, R. Ehricht, and D. C. Coleman.** 2011. Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent *mecA*, *mecI*, *mecR1*, blaZ, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **55:**3765-73.
321. **Sieradzki, K., T. Leski, J. Dick, L. Borio, and A. Tomasz.** 2003. Evolution of a vancomycin-intermediate *Staphylococcus aureus* strain in vivo: multiple changes in the antibiotic resistance phenotypes of a single lineage of methicillin-resistant *S. aureus* under the impact of antibiotics administered for chemotherapy. J Clin Microbiol **41:**1687-93.
322. **Sieradzki, K., R. B. Roberts, S. W. Haber, and A. Tomasz.** 1999. The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. N Engl J Med **340:**517-23.
323. **Sievert, D. M., J. T. Rudrik, J. B. Patel, L. C. McDonald, M. J. Wilkins, and J. C. Hageman.** 2008. Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002-2006. Clin Infect Dis **46:**668-74.
324. **Simoes, R. R., M. Aires-de-Sousa, T. Conceicao, 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.
325. **Sinha, B., P. P. Francois, O. Nusse, M. Foti, O. M. Hartford, P. Vaudaux, T. J. Foster, D. P. Lew, M. Herrmann, and K. H. Krause.** 1999. Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. Cell Microbiol **1:**101-17.
326. **Sinha, B., and M. Fraunholz.** 2010. *Staphylococcus aureus* host cell invasion and post-invasion events. Int J Med Microbiol **300:**170-5.

327. **Sissolak, D., A. Geusau, G. Heinze, W. Witte, and M. L. Rotter.** 2002. Risk factors for nasal carriage of *Staphylococcus aureus* in infectious disease patients, including patients infected with HIV, and molecular typing of colonizing strains. Eur J Clin Microbiol Infect Dis **21:**88-96.
328. **Skinner, D., and C. S. Keefer. .** 1941. Significance of bacteremia caused by *Staphylococcus aureus*. Arch. Intern. Med **68:**851-875.
329. **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.
330. **Smith, T. C., and N. Pearson.** 2011. The emergence of *Staphylococcus aureus* ST398. Vector Borne Zoonotic Dis **11:**327-39.
331. **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*. Glycopeptide-Intermediate *Staphylococcus aureus* Working Group. N Engl J Med **340:**493-501.
332. **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, P. H. Van, and A. S. 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.
333. **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.
334. **Stam-Bolink, E. M., D. Mithoe, W. H. Baas, J. P. Arends, and A. V. Moller.** 2007. Spread of a methicillin-resistant *Staphylococcus aureus* ST80 strain in the community of the northern Netherlands. Eur J Clin Microbiol Infect Dis **26:**723-7.
335. **Stegger, M., J. A. Lindsay, M. Sorum, 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. **Struelens, M. J.** 1996. Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. Clinical Microbiology and Infection **2:** 2–11.
337. **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.
338. **Szmigielski, S., E. Sobiczewska, G. Prevost, H. Monteil, D. A. Colin, and J. Jeljaszewicz.** 1998. Effect of purified staphylococcal leukocidal toxins on isolated blood polymorphonuclear leukocytes and peritoneal macrophages in vitro. Zentralbl Bakteriol **288:**383-94.
339. **Tacconelli, E., Y. Carmeli, A. Aizer, G. Ferreira, M. G. Foreman, and E. M. D'Agata.** 2003. Mupirocin prophylaxis to prevent *Staphylococcus aureus* infection in patients undergoing dialysis: a meta-analysis. Clin Infect Dis **37:**1629-38.
340. **Takahashi, T., I. Satoh, and N. Kikuchi.** 1999. Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on *16S rRNA* gene sequence analysis. Int J Syst Bacteriol **49 Pt 2:**725-8.
341. **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.
342. **Takano, T., W. Higuchi, H. Zaraket, 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.

343. **Tan, Y. K., K. L. Khoo, S. P. Chin, and Y. Y. Ong.** 1998. Aetiology and outcome of severe community-acquired pneumonia in Singapore. Eur Respir J **12:**113-5.
344. **Tavares, D. A., R. Sa-Leao, M. Miragaia, and H. de Lencastre.** 2010. Large screening of CA-MRSA among *Staphylococcus aureus* colonizing healthy young children living in two areas (urban and rural) of Portugal. BMC Infect Dis **10:**110.
345. **Teixeira, L. A., C. A. Resende, L. R. Ormonde, R. Rosenbaum, A. M. Figueiredo, H. de Lencastre, and A. Tomasz.** 1995. Geographic spread of epidemic multiresistant *Staphylococcus aureus* clone in Brazil. J Clin Microbiol **33:**2400-4.
346. **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.
347. **Tenover, F. C., S. McAllister, G. Fosheim, L. K. McDougal, R. B. Carey, B. Limbago, D. Lonsway, J. B. Patel, M. J. Kuehnert, and R. Gorwitz.** 2008. Characterization of *Staphylococcus aureus* isolates from nasal cultures collected from individuals in the United States in 2001 to 2004. J Clin Microbiol **46:**2837-41.
348. **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.
349. **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.
350. **Thomas, B., A. Pugalenthi, and M. Chilvers.** 2009. Pleuropulmonary complications of PVL-positive *Staphylococcus aureus* infection in children. Acta Paediatr **98:**1372-5.
351. **Tinelli, M., M. Monaco, M. Vimercati, A. Ceraminiello, and A. Pantosti.** 2009. Methicillin-susceptible *Staphylococcus aureus* in skin and soft tissue infections, Northern Italy. Emerg Infect Dis **15:**250-7.
352. **Tomasz, A., H. B. Drugeon, H. M. de Lencastre, D. Jabes, L. McDougall, and J. Bille.** 1989. 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.
353. **Tristan, A., M. Bes, H. Meugnier, G. Lina, B. Bozdogan, P. Courvalin, M. E. Reverdy, M. C. Enright, F. Vandenesch, and J. Etienne.** 2007. Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant *Staphylococcus aureus*, 2006. Emerg Infect Dis **13:**594-600.
354. **Tweten, R. K., K. K. Christianson, and J. J. Iandolo.** 1983. Transport and processing of staphylococcal alpha-toxin. J Bacteriol **156:**524-8.
355. **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.
356. **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**.
357. **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.
358. **Valentini, P., G. Parisi, M. Monaco, F. Crea, T. Spanu, O. Ranno, M. Tronci, and A. Pantosti.** 2008. An uncommon presentation for a severe invasive infection due to methicillin-resistant *Staphylococcus aureus* clone USA300 in Italy: a case report. Ann Clin Microbiol Antimicrob **7:**11.
359. **Valeva, A., M. Palmer, and S. Bhakdi.** 1997. Staphylococcal alpha-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. Biochemistry **36:**13298-304.

360. **van Belkum, A., D. C. Melles, J. Nouwen, W. B. van Leeuwen, W. van Wamel, M. C. Vos, H. F. Wertheim, and H. A. Verbrugh.** 2009. Co-evolutionary aspects of human colonisation and infection by *Staphylococcus aureus*. Infect Genet Evol **9:**32-47.

361. **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.** 2008. Methicillin-resistant and -susceptible *Staphylococcus aureus* sequence type 398 in pigs and humans. Emerg Infect Dis **14:**479-83.

362. **van Belkum, A., H. G. Niesters, W. G. MacKay, and W. B. van Leeuwen.** 2007. Quality control of direct molecular diagnostics for methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **45:**2698-700.

363. **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.

364. **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. Paszti, 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.

365. **van der Mee-Marquet, N., D. Hernandez, X. Bertrand, R. Quentin, A. R. Corvaglia, and P. Francois.** 2013. Whole-Genome Sequence of the Ancestral Animal-Borne ST398 *Staphylococcus aureus* Strain S123. Genome Announc **1**.

366. **van Wamel, W., Y. Q. Xiong, A. S. Bayer, M. R. Yeaman, C. C. Nast, and A. L. Cheung.** 2002. Regulation of *Staphylococcus aureus* type 5 capsular polysaccharides by *agr* and *sarA* in vitro and in an experimental endocarditis model. Microb Pathog **33:**73-9.

367. **Vandenbergh, M. F., and H. A. Verbrugh.** 1999. Carriage of *Staphylococcus aureus*: epidemiology and clinical relevance. J Lab Clin Med **133:**525-34.

368. **Vandendriessche, S., M. Hallin, B. Catry, B. Jans, A. Deplano, C. Nonhoff, S. Roisin, R. De Mendonca, M. J. Struelens, and O. Denis.** 2012. Previous healthcare exposure is the main antecedent for methicillin-resistant *Staphylococcus aureus* carriage on hospital admission in Belgium. Eur J Clin Microbiol Infect Dis **31:**2283-92.

369. **Vandendriessche, S., K. Kadlec, S. Schwarz, and O. Denis.** 2011. Methicillin-susceptible *Staphylococcus aureus* ST398-t571 harbouring the macrolide-lincosamide-streptogramin B resistance gene erm(T) in Belgian hospitals. J Antimicrob Chemother **66:**2455-9.

370. **Vandenesch, F., J. Kornblum, and R. P. Novick.** 1991. A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J Bacteriol **173:**6313-20.

371. **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.

372. **Verkade, E., T. Bosch, Y. Hendriks, and J. Kluytmans.** 2012. Outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch nursing home. Infect Control Hosp Epidemiol **33:**624-6.

373. **Vogel, V., L. Falquet, S. P. Calderon-Copete, P. Basset, and D. S. Blanc.** 2012. Short term evolution of a highly transmissible methicillin-resistant *Staphylococcus aureus* clone (ST228) in a tertiary care hospital. PLoS One **7:**e38969.

374. **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.

375. **Voyich, J. M., K. R. Braughton, D. E. Sturdevant, A. R. Whitney, B. Said-Salim, S. F. Porcella, R. D. Long, D. W. Dorward, D. J. Gardner, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo.** 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol **175:**3907-19.

376. **Walker, B., and H. Bayley.** 1995. Key residues for membrane binding, oligomerization, and pore forming activity of staphylococcal alpha-hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. J Biol Chem **270:**23065-71.

377. **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.

378. **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:**1510-4.

379. **Wang, W. Y., T. S. Chiueh, J. R. Sun, S. M. Tsao, and J. J. Lu.** 2012. Molecular typing and phenotype characterization of methicillin-resistant *Staphylococcus aureus* isolates from blood in Taiwan. PLoS One **7:**e30394.

380. **Wannet, W. J., E. Spalburg, M. E. Heck, G. N. Pluister, E. Tiemersma, R. J. Willems, X. W. Huijsdens, A. J. de Neeling, and J. Etienne.** 2005. Emergence of virulent methicillin-resistant *Staphylococcus aureus* strains carrying Panton-Valentine leucocidin genes in The Netherlands. J Clin Microbiol **43:**3341-5.

381. **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.

382. **Weigel, L. M., R. M. Donlan, D. H. Shin, B. Jensen, N. C. Clark, L. K. McDougal, W. Zhu, K. A. Musser, J. Thompson, D. Kohlerschmidt, N. Dumas, R. J. Limberger, and J. B. Patel.** 2007. High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. Antimicrob Agents Chemother **51:**231-8.

383. **Weist, K., K. Pollege, I. Schulz, H. Ruden, and P. Gastmeier.** 2002. How many nosocomial infections are associated with cross-transmission? A prospective cohort study in a surgical intensive care unit. Infect Control Hosp Epidemiol **23:**127-32.

384. **Welinder-Olsson, C., K. Floren-Johansson, L. Larsson, S. Oberg, L. Karlsson, and C. Ahren.** 2008. Infection with Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* t034. Emerg Infect Dis **14:**1271-2.

385. **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.

386. **Wertheim, H. F., M. C. Vos, A. Ott, A. van Belkum, A. Voss, J. A. Kluytmans, P. H. van Keulen, C. M. Vandenbroucke-Grauls, M. H. Meester, and H. A. Verbrugh.** 2004. Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers. Lancet **364:**703-5.

387. **Whitby, M., M. L. McLaws, and G. Berry.** 2001. Risk of death from methicillin-resistant *Staphylococcus aureus* bacteraemia: a meta-analysis. Med J Aust **175:**264-7.

388. **Whitener, C. J., S. Y. Park, F. A. Browne, L. J. Parent, K. Julian, B. Bozdogan, P. C. Appelbaum, J. Chaitram, L. M. Weigel, J. Jernigan, L. K. McDougal, F. C. Tenover, and S. K. Fridkin.** 2004. Vancomycin-resistant *Staphylococcus aureus* in the absence of vancomycin exposure. Clin Infect Dis **38:**1049-55.

389. **Wiener-Well, Y., M. Galuty, B. Rudensky, Y. Schlesinger, D. Attias, and A. M. Yinnon.** 2011. Nursing and physician attire as possible source of nosocomial infections. Am J Infect Control **39:**555-9.

390. **Williams, J. V., B. R. Vowels, P. J. Honig, and J. J. Leyden.** 1998. *S. aureus* isolation from the lesions, the hands, and the anterior nares of patients with atopic dermatitis. Pediatr Dermatol **15:**194-8.
391. **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:**414-422.
392. **Witte, W., B. Strommenger, C. Cuny, D. Heuck, and U. Nuebel.** 2007. Methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006. J Antimicrob Chemother **60:**1258-63.
393. **Witte, W., B. Strommenger, I. Klare, and G. Werner.** 2004. [Antibiotic-resistant nosocomial pathogens. Part I: diagnostic and typing methods]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz **47:**352-62.
394. **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.
395. **Wright, G. D.** 2005. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv Drug Deliv Rev **57:**1451-70.
396. **Xiong, Y. Q., J. Willard, M. R. Yeaman, A. L. Cheung, and A. S. Bayer.** 2006. Regulation of *Staphylococcus aureus* alpha-toxin gene (*hla*) expression by *agr*, *sarA*, and *sae* in vitro and in experimental infective endocarditis. J Infect Dis **194:**1267-75.
397. **Yang, E. S., J. Tan, S. Eells, G. Rieg, G. Tagudar, and L. G. Miller.** 2010. Body site colonization in patients with community-associated methicillin-resistant *Staphylococcus aureus* and other types of S. aureus skin infections. Clin Microbiol Infect **16:**425-31.
398. **Yao, D., F. Y. Yu, Z. Q. Qin, C. Chen, S. S. He, Z. Q. Chen, X. Q. Zhang, and L. X. Wang.** 2010. Molecular characterization of *Staphylococcus aureus* isolates causing skin and soft tissue infections (SSTIs). BMC Infect Dis **10:**133.
399. **Young, B. C., T. Golubchik, E. M. Batty, R. Fung, H. Larner-Svensson, A. A. Votintseva, R. R. Miller, H. Godwin, K. Knox, R. G. Everitt, Z. Iqbal, A. J. Rimmer, M. Cule, C. L. Ip, X. Didelot, R. M. Harding, P. Donnelly, T. E. Peto, D. W. Crook, R. Bowden, and D. J. Wilson.** 2012. Evolutionary dynamics of *Staphylococcus aureus* during progression from carriage to disease. Proc Natl Acad Sci U S A **109:**4550-5.
400. **Yu, F., Z. Chen, C. Liu, X. Zhang, X. Lin, S. Chi, T. Zhou, Z. Chen, 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.
401. **Yu, V. L., A. Goetz, M. Wagener, P. B. Smith, J. D. Rihs, J. Hanchett, and J. J. Zuravleff.** 1986. *Staphylococcus aureus* nasal carriage and infection in patients on hemodialysis. Efficacy of antibiotic prophylaxis. N Engl J Med **315:**91-6.
402. **Zecconi, A., and F. Scali.** 2013. *Staphylococcus aureus* virulence factors in evasion from innate immune defenses in human and animal diseases. Immunol Lett **150:**12-22.
403. **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.
404. **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.
405. **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.
406. **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.

407. **Zhang, X., Q. Hu, W. Yuan, W. Shang, H. Cheng, J. Yuan, J. Zhu, Z. Hu, S. Li, W. Chen, X. Hu, and X. Rao.** 2013. First report of a sequence type 239 vancomycin-intermediate *Staphylococcus aureus* isolate in Mainland China. Diagn Microbiol Infect Dis **77:**64-8.

408. **Zinderman, C. E., B. Conner, M. A. Malakooti, J. E. LaMar, A. Armstrong, and B. K. Bohnker.** 2004. Community-acquired methicillin-resistant *Staphylococcus aureus* among military recruits. Emerg Infect Dis **10:**941-4.

CHAPTER II

High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in the community in Portugal:
evidence for the blurring of community-hospital boundaries

THIS CHAPTER WAS PUBLISHED IN:

Tavares, A., M. Miragaia, J. Rolo, C.Coelho, H. de Lencastre and CA-MRSA/MSSA working group. 2013. High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in the community in Portugal: evidence for the blurring of community hospital boundaries. Eur. J. Clin. Microbiol. Infect. Dis. 32:1269-83.

ABSTRACT

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of infection in the community (CA-MRSA), but in spite of its relevance, no data exist concerning its epidemiology in Portugal. In this study, we aimed to evaluate the prevalence, population structure, and origin of MRSA in the Portuguese community.

Methods

A total of 527 isolates, both methicillin-susceptible *S. aureus* (MSSA) and MRSA, were collected from individuals with no healthcare related risk factors attending 16 healthcare institutions in Portugal. Isolates were characterized for the presence of *mec*A, Panton–Valentine leukocidin (PVL), and arginine catabolic mobile element (ACME), and by staphylococcal cassette chromosome *mec* (SCC*mec*) typing, pulsed-field gel electrophoresis (PFGE), spa, and multilocus sequence typing (MLST). Susceptibility to a panel of 13 antibiotics was tested. Isolates relatedness was analyzed by goeBURST and BURP.

Results

We found a high frequency (21.6%) of MRSA in the community. However, only 11.4% of the isolates belonged to typical CA-MRSA epidemic clones (USA300, USA400, USA700, Southwest Pacific, European, and ST398). The remaining isolates, which constituted the great majority (88.6%), belonged to hospital-associated MRSA (HA-MRSA) epidemic clones, namely, to the EMRSA-15 clone (77.2%). PVL was rare and carried by 17 isolates only (five MRSA and 12 MSSA). In the whole collection, some MRSA and MSSA were highly related.

Conclusion

The high frequency of MRSA in the community in Portugal seems to result mainly from dissemination from the hospital. They might also have emerged from an extant MSSA population, by SCC*mec* acquisition, or MRSA clonal introduction from abroad.

INTRODUCTION

Although methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be considered one of the major hospital-associated pathogens (HA-MRSA), in recent years, MRSA has been increasingly reported worldwide as a causative agent of infections in healthy individuals in the community (CA-MRSA).

CA-MRSA first emerged in five main genetic backgrounds (e.g., ST1, ST8, ST30, ST72, ST80), which were different from those found in the hospital. CA-MRSA has been recognized as more susceptible to antibiotics other than betalactams, to carry smaller size staphylococcal cassette chromosome *mec* (SCC*mec*) (types IV and V), and to be frequently associated with the presence of the Panton–Valentine leukocidin (PVL) (20). Moreover, CA-MRSA has been described to have an improved virulence capacity, which is believed to be associated to the presence and differential expression of specific virulence factors, e.g., PVL, arginine catabolic mobile element (ACME), α-hemolysin (Hla), and α-phenol soluble modulins (PSMs) (13, 53, 76, 77).

The main CA-MRSA genetic backgrounds emerged and developed differently in separate geographical areas. The first CA-MRSA episodes were described in 1993 in remote communities in Western Australia (73), associated to the Southwest Pacific clone (SWP) (ST30-IVc) (18). Six years later, in the

USA, the death of four children was reported to be caused by isolates belonging to the USA400 clone (ST1-IVa) (5). Nevertheless, since then, outbreaks of infections in the USA were mainly associated to a different clone, the USA300 clone (ST8-IVa) (39), that quickly overcame the USA400 clone. On the other hand, in Europe, the so-called European clone (ST80-IVc) emerged, with the first outbreaks of CA-MRSA being reported in Greece (7). Later, in Europe, MRSA emerged also in livestock. This corresponded mainly to a single clonal type (ST398-IV/V), associated to colonization in pigs. The first descriptions were reported in France (11), but, nowadays, the ST398 clone is disseminated worldwide, not only in animals but also in humans (24, 38, 74).

Presently, more than 20 distinct genetic lineages of CA-MRSA are known (47), but their epidemicity varies from clone to clone. One of the most epidemic CA-MRSA clones is USA300, which has already been reported in as many as 50 different countries (56, 65). However, it has much higher clinical impact in the USA than in Europe (e.g., the UK, Spain, Switzerland, France), Asia (Japan, Hong Kong), and Australia, where this clone is seen only sporadically (52). The most relevant clone

in Europe remains the European clone, having been isolated in every European country sampled to date (65), although particular clinical problems were registered predominantly in Greece, but also in Denmark and the Netherlands (52). In recent years, the European clone was reported in the Middle East (Kuwait, Lebanon, Israel, Egypt, Algeria, Tunisia) and also in Australia (47, 52), showing some transcontinental spread. Another clone that has a high epidemicity is the SWP clone that was found in Germany, UK, Switzerland, Scandinavia, Hong Kong, Taiwan, Ireland, Kuwait, and USA (47, 52). Finally, ST398 initially found in the northern European countries, Denmark and Netherlands, was reported later in several other European countries, Canada and Australia, in pigs and humans, and, more recently, in a broader range of animals (cattle, horses, dogs, poultries, chickens, turkeys) (14, 40).

In general, there is an asymmetry in the CA-MRSA epidemiology between the USA and the rest of the world. Whereas in the USA, the prevalence of community-associated MRSA infections is very high, reaching 59% (54), and a single clone (USA300) was found, in Europe, the prevalence of CA-MRSA continues to be relatively low, ranging from 1 to 29% (42, 44, 45, 79), and its population structure is composed by several different CA-MRSA epidemic clones and variants of these clones (65).

In Portugal, the European country with the highest rate of MRSA in nosocomial infections, national surveillance has been conducted over the years exclusively in hospitals (8). In spite of the clinical relevance of CA-MRSA, little information exists on MRSA epidemiology outside the nosocomial setting. The previous screening of MRSA in the nasopharynx of young children attending daycare centers (66, 71) and in the anterior nares of military draftees, nonmedical university students, and high-school students (66) indicated an extremely low prevalence of MRSA carriage (<1%), associated to the ST82 and USA700 clone. Moreover, four infection episodes were described to be caused by isolates belonging to the European (17), USA300 (55) and ST398 (63) clones.

In this study we developed a structured survey with the aim of evaluating the prevalence, origin and main clones of MRSA circulating in the community in Portugal.

MATERIAL AND METHODS

Ethical Statement

Isolates were obtained as part of a project that aims to monitor the relevance of the CA-MRSA causing infections in the Portuguese community. All data were collected in accordance with the European Parliament and Council decision for the epidemiological surveillance and control of communicable disease in the European community (2, 3) where personnel ethical approval and informed consent is not required.

Study design

Between January 2009 and September 2010, a total of 1,487 *S. aureus* isolates were collected from infection and colonization, from single patients attending 16 Portuguese healthcare institutions (14 hospitals, one clinical laboratory, and one healthcare center) located in different geographic regions of Portugal, covering the North, Center, and South. All collaborating institutions were asked to fill in a questionnaire for each isolate collected. The great majority of isolates (85%, 1,267 out of 1,487) were accompanied by the respective questionnaires. Questionnaires data were introduced into a dedicated database in the research laboratory [Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB)]. From the 1,267 isolates, 527 (41.6%) were defined as having a community origin and were included in this study (see the next section). The remaining 740 (58.4%) isolates were classified as having an hospital origin, since they were collected after 48 h of hospitalization and/or from individuals with at least one risk factor for MRSA infection or colonization. These 740 isolates were not characterized further in this work.

Questionnaires structure and community-associated MRSA definition

Questionnaires were designed based on the Centers for Disease Control and Prevention (CDC) epidemiological definition used to distinguish CA-MRSA from HA-MRSA infections (http://www.cdc.gov/). In addition questions were included addressing patient demographic information (age, gender) and general clinical and microbiological data (biological specimens, clinical relevance, clinical diagnosis and antibiotic susceptibility profile data).

Isolates were considered to be CA-MRSA if they were collected from outpatients (patients attending healthcare centers and clinical laboratories) or from inpatients (within 48 h of hospital admission) and did not have risk factors for HA-MRSA acquisition, namely, recent hospitalization, surgery,

dialysis, presence of indwelling devices, history of MRSA infection or colonization in the past year, and a regular contact with a geriatric daycare center.

Antimicrobial susceptibility testing

The antimicrobial susceptibility was tested in the collaborating institutions using the Vitek 2 system (bioMérieux, Inc., Durham, NC) for a panel of 13 antibiotics: penicillin (PEN), oxacillin (OXA), vancomycin (VAN), linezolid (LNZ), gentamicin (GEN), ciprofloxacin (CIP), erythromycin (ERY), clindamycin (CLI), quinupristin/dalfopristin (QDA), tetracycline (TET), rifampicin (RIF), fusidic acid (FUS), and trimethoprim–sulfamethoxazole (SXT). Information on susceptibility to some antimicrobial agents was not provided by all healthcare institutions for several isolates. In the case of OXA-resistant isolates (112 out of 527), the missing antimicrobial susceptibility data were completed by performing disk diffusion tests according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (1). Isolates that presented resistance to more than three classes of antibiotics were classified as having a multidrug resistance profile. For OXA-susceptible isolates (415 out of 527), only 73 isolates (17.6%) were tested against all antibiotics by the healthcare institutions.

Detection of *mecA* and PVL

The presence of *mecA* and PVL genes (*lukS*-PV/ *lukF*-PV) was detected by polymerase chain reaction (PCR) amplification in all isolates, as previously described (43, 58). Isolates were considered as MRSA when they carried *mecA* and as MSSA when *mecA* was absent.

Determination of type of SCC*mec*

For MRSA isolates, the structure of the SCC*mec* was determined by a multiplex PCR using specific primers to each SCC*mec* type (51). If isolates were non-typeable by this method, SCC*mec* typing was performed based on the amplification of the *mec* and *ccr* complexes by PCR (35, 57, 59). The subtype of SCC*mec* IV was determined by multiplex PCR developed by Milheiriço *et al.* (50).

PFGE

Pulsed-field gel electrophoresis (PFGE) was performed for all isolates, as previously described (15). The *SmaI* restriction bands patterns were compared with band patterns of reference CA- and HA-

MRSA epidemic control strains by using the BioNumerics software version 4.61 (Applied Maths, Saint-Martens-Latem, Belgium) with previously defined settings (optimization: 0.5%, tolerance: 1.3%, cut-off: 80%) (25). All isolates that clustered with more than 80% similarity (PFGE type) with the PFGE band pattern profile of CA and HA-MRSA epidemic control strains were not further characterized at molecular level. In this case, the final clonal classification was based on the association of PFGE type and SCC*mec* data for MRSA and only PFGE type for MSSA.

The isolates that were resistant to digestion with *SmaI* were classified as a putative livestock-associated clone ST398, since this characteristic is specific of this clone (12).

spa typing

spa typing was performed by a PCR amplification and sequencing of a polymorphic region of the *S. aureus* protein A gene (*spa* gene) (67) for isolates containing a similarity below 80% with the restriction band pattern of CA or HA-MRSA epidemic control strains and isolates resistant to digestion with *SmaI*. The *spa* types were assigned using the RIDOM web server (http://spaserver.ridom.de). The BURP algorithm was used in order to cluster groups of related *spa* types (48).

MLST

The sequence type (ST) was inferred using *spa* server (http://spaserver.ridom.de) and/or data obtained from the literature. In the case the STs could not be predicted based on the *spa* type, multilocus sequence typing (MLST) was performed as previously described (23). The alleles for each gene and the ST for each allelic profile were attributed according to the MLST database (http://www.mlst.net/). To evaluate the population structure and patterns of evolution, the algorithm goeBURST (http://goeburst.phyloviz.net/) was used.

Detection of ACME

Arginine catabolic mobile element (ACME) allotypes (type I to III) were defined based on multiplex PCR amplification using primers specific to the loci *arc* and *opp3*, as previously described (22). The presence of ACME was tested exclusively in the MRSA isolates belonging to the USA300 clone (21, 22).

Clone definition: epidemic, epidemic-related, and minor clone

Clones were defined based on the genotypic features found for each isolate. Isolates were defined as belonging to an **epidemic clone** if they contained the same genetic background by PFGE type and carried the same SCC*mec* as the reference epidemic control strains. Isolates were considered to be **related to an epidemic clone** if they contained at least the PFGE type or the *spa* type similar to the prototype strain. Isolates were considered as **minor clones** if they were not related neither by PFGE type nor by *spa* type to the reference epidemic clones.

Statistical analysis

The comparison of groups of categorical data was analyzed using the $\chi 2$ test with a level of significance set at 0.05. The degree of genetic diversity was estimated for MRSA/MSSA by the Simpsons's index of diversity (SID) using the confidence interval of 95% and defining as groups the combination of *spa* type and ST (70).

RESULTS

Definition of *S. aureus* isolates origin

From the 527 isolates included in this study, 141 (26.8%) followed strictly the CDC epidemiological definition of isolates with community origin (community isolates); the other 386 (73.2%) were collected from people with no risk factors for healthcare contact, but since not all information requested in the questionnaires was available, possible risk factors for MRSA infection/colonization could not be ruled out (community-onset).

The results obtained by the combination of all typing methods showed that no significant difference was observed in the distribution of HA and CA genetic backgrounds between these two groups [community: HA backgrounds, 63.1% (89 isolates)/CA backgrounds, 36.9% (52 isolates); community-onset: HA, 55.7% (215 isolates)/CA, 44.3% (171 isolates)] (p=0.13). For this reason, all 527 isolates were considered as having a community-onset origin.

Frequency and population structure of MRSA in the community

Among the 527 isolates with a community origin, a significant proportion was MRSA (21.6%, n=114). However, contrarily to what was expected, the great majority (88.6%, n=101) belonged or was related to typical HA-MRSA epidemic clones and only a small fraction (11.4%, n=13) belonged to CA-MRSA epidemic clones (Table 1). PVL was rarely found in MRSA backgrounds (29.4%, n=5) and was present exclusively in isolates belonging to the USA300 clone.

Among the 101 isolates related or belonging to HA genetic backgrounds, the great majority belonged to EMRSA-15 or related clones (ST22-IVh, ST1806-IVh, t032, t020, t3212, t670, t036, t7980, t747) (77.2%, n=78) and New York/Japan (NY/JP) or related clones (ST5-II, ST105-II, t005) (14.9%, n=15). The remaining isolates belonged to the Pediatric clone (ST5-IVc/VI, t311, t179, t002) (5.9%, n=6), EMRSA-16 clone (ST36-II, t018) (1%, n=1), and Brazilian clone, represented by a single locus variant (SLV) of ST239 (ST2246–III, t037) (1%, n=1).

A high genetic diversity was observed among the 13 CAMRSA isolates: five isolates belonged or were related to the USA300 clone [ST8-IVa, t008, PVL (5), ACME I (4), ACME II (1)], two isolates belonged to ST398 (ST398-IVa, t011, t1451), two isolates to the SWP clone (ST30-IVa, t012), and the remaining four isolates to the USA700 (ST72-IVg, t148), European (ST80-IV_{NT}, t044), USA400 (ST1-IVa, t127), and a SLV of ST72 (ST1810-IVa, t1346) (one isolate each).

Among all 114 MRSA isolates characterized in this study, we found a total of 20 different spa types and 13 STs (Table 1) clustered in 11 clonal complexes (CCs) (data not shown). The most frequent SCC*mec* was SCC*mec* IV (84.2%, n=96), **f**ollowed by SCC*mec* II (14%, n=16), SCC*mec* VI (0.9%, n=1), and SCC*mec* III (0.9%, n=1). The most predominant SCC*mec* IV subtype was SCC*mec* IVh (54.2%, n=52), followed by IVa (10.4%, n=10), IVc (7.3%, n=7), IVg (1%, n=1), and for 26 isolates (27.1%), the subtypes of SCC*mec* IV were non-typeable by the multiplex strategy used.

Table 1. Molecular characterization of the 114 MRSA isolates analyzed in this study.

Clonal type (no isolates)	**MLST**	**SCC*mec* type/ SCC*mec* IV subtype**	***spa* type**	**Relatedness with clone (PFGE)** (no isolates)	**PVL**	**Antibiotic resistance profile** (no isolates)
HA-MRSA background (101 isolates)						
EMRSA-15/ EMRSA-15 related (78)	ST22; ST1806 (TLV ST22)	IVh	t032, t020; t3212; t670; t036; t7980; t747	EMRSA-15 (69); Minor (9)	negative	CIP-ERY-CLI (39)^{M}; CIP (19); CIP-ERY-CLI^{I}(6)^{M}; CIP-ERY (4); CIP-ERY-CLI^{I}- RIF (1); CIP-ERY-RIF (1); GEN-CIP (1); CIP-ERY-TET (1); CIP-RIF (1); CIP-ERY-CLI-GEN-RIF-TET^{I} (1)^{M}
NY/JP/ NY/JP related (15)	ST5; ST105 (SLV ST5)	II	t002	NY/JP (13); Minor (2)	negative	CIP-ERY-CLI (7)^{M}; CIP-ERY-CLI-RIF (1)^{M}; CIP-ERY-CLI-TET-RIF (1)^{M}; CIP-ERY (4); CIP-ERY-CLI-FUS^{I} (1)^{M}; CIP-ERY-CLI-TET-RIF^{I}-FUS^{I} (1)^{M}
Pediatric/ Pediatric related (6)	ST5	IVc/ VI	t311; t179; t002	Pediatric (2); Minor (4)	negative	CIP-ERY-CLI-GEN (1)^{M}; CIP-ERY-CLI (1)^{M}; CIP (1); FUS^{I} (1)
EMRSA-16 (1)	ST36	II	t018	EMRSA-16 (1)	negative	CIP-ERY-CLI (1)
Brazilian related (1)	ST2246 (SLV ST239)	III	t037	Minor (1)	negative	CIP-ERY-CLI-GEN-TET-RIF-SXT (1)^{M}
CA-MRSA background (13 isolates)						
USA300/ USA300 related (5)	ST8	IVa	t008	USA300 (4); Minor (1)	**positive**	CIP-ERY (4); CIP-ERY-CLI (1)^{M}
ST398 (2)	ST398	IVa	t011; t1451	no SmaI resctriction	negative	GEN-TET (1); GEN-TET-CLI^{I}(1)^{M}
SWP/ SWP related (2)	ST30	IVa	t012	SWP (1); Minor (1)	negative	CIP^{I}(1)
USA700 (1)	ST72	IVg	t148	USA700 (2)	negative	-
European (1)	ST80	IV $_{NT}$	t044	European	negative	TET (1)
USA400 (1)	ST1	IVa	t127	USA400	negative	ERY-CLI (1)
ST1810-IVa (1)	ST1810 (SLV 72)	IVa	t1346	USA700	negative	-

(I) Intermediate Resistance; (M) Multidrug-resistance profile: resistant to more than three classes of antibiotics, other than beta-lactams; CIP– ciprofloxacin; CLI – clindamycin; ERY – erythromycin; GEN – gentamicin; TET – tetracycline; RIF – rifampicin.

Characteristics of the population and biological specimens from which MRSA were isolated

The population from which MRSA was isolated was composed of 59 males and 54 females. For one individual, no information was available regarding the gender. MRSA was found in a total of 13 children (0–18 years old), 44 adults (19–64 years old), and 56 elderly (>65 years old); no information was available regarding the age for one individual.

Isolates were recovered from patients attending hospital medical care as outpatients (n=33) or inpatients (<48 h) (n=30), in the emergency room (n=28), and also from patients attending a clinical laboratory (n=21) and a healthcare center (n=2). Most of the isolates were from infection samples (87.7%, n=100), and few were from colonization (10.5%, n=12). For the remaining two isolates (1.8%), no information was available regarding the clinical significance of the specimen.

MRSA were collected mainly from purulent exudates (47.4%, n=54) and respiratory secretions (14%, n=16), but also from blood (3.5%, n=4), urine (2.6%, n=3), and others (23.7%, n=27). In 10 isolates (8.8%), in which the information on the type of clinical product was not available, four isolates were from infection and five isolates were from colonization. For a single isolate, neither information on the clinical product nor on clinical significance was available.

CA-MRSA more likely caused general bloodstream infections (15.4%) than HA-MRSA (2.6%) (p=0.01). No other significant difference was registered between CA-MRSA *versus* HA-MRSA backgrounds (Supplemental Table S1).

MRSA antimicrobial resistance profiles

A high percentage of isolates belonging to HA-MRSA clones showed a multidrug resistance profile (64.4%, 65 out of 101). The most predominant resistance profile included resistance to PEN, OXA, CIP, ERY, and CLI (48.5%, 49 out of 101), and was mainly observed in isolates belonging or related to EMRSA-15 (85.7%, 42 out of 49), but also to the NY/JP clone (14.3%, 7 out of 49).

As expected, isolates belonging to CA-MRSA clones were shown to be highly susceptible, although two out of the 13 isolates found were multidrug-resistant. These isolates belonged to USA300 and ST398 and were resistant to PEN, OXA, CIP, ERY, CLI and resistant to PEN, OXA, GEN, TET, CLI^{Int}, respectively.

Geographic distribution of MRSA clones

The highest MRSA prevalence was found in the district of Oporto, with a frequency of 25%, followed by Lisbon and Braga, both with a prevalence of 21%, and Coimbra and Algarve, with prevalences of 19% and 5%, respectively (Figure 1, b).

There was a high genetic diversity in the MRSA clonal types found in each geographic region (Figure 1, b). In the northern region, represented by Braga and Oporto, we observed that the dominant clone in the community was EMRSA-15 (78.3% and 83.6%, respectively). Less than 10% of the MRSA population belonged to several different CA epidemic clones in this region. The only exception was the MRSA ST398 clone that was associated exclusively to individuals living in a rural area near Oporto (Santo Tirso). In the center region of Portugal, Coimbra, the HA clones present in the community were equally represented by the EMRSA-15 (40%) and NY/JP clones (40%), and no CA clones were found. In Lisbon, a different scenario was observed. In contrast to the other regions, the proportion of NY/JP (36.8%) clones was slightly higher than EMRSA-15 (21.1%). Moreover, the CA clones were well represented (31.6%), mostly by USA300 (83.3%) and USA700 related clones (16.7%). Finally, in the Southern region, Algarve, only one isolate (belonging to the USA700 clone) was recovered.

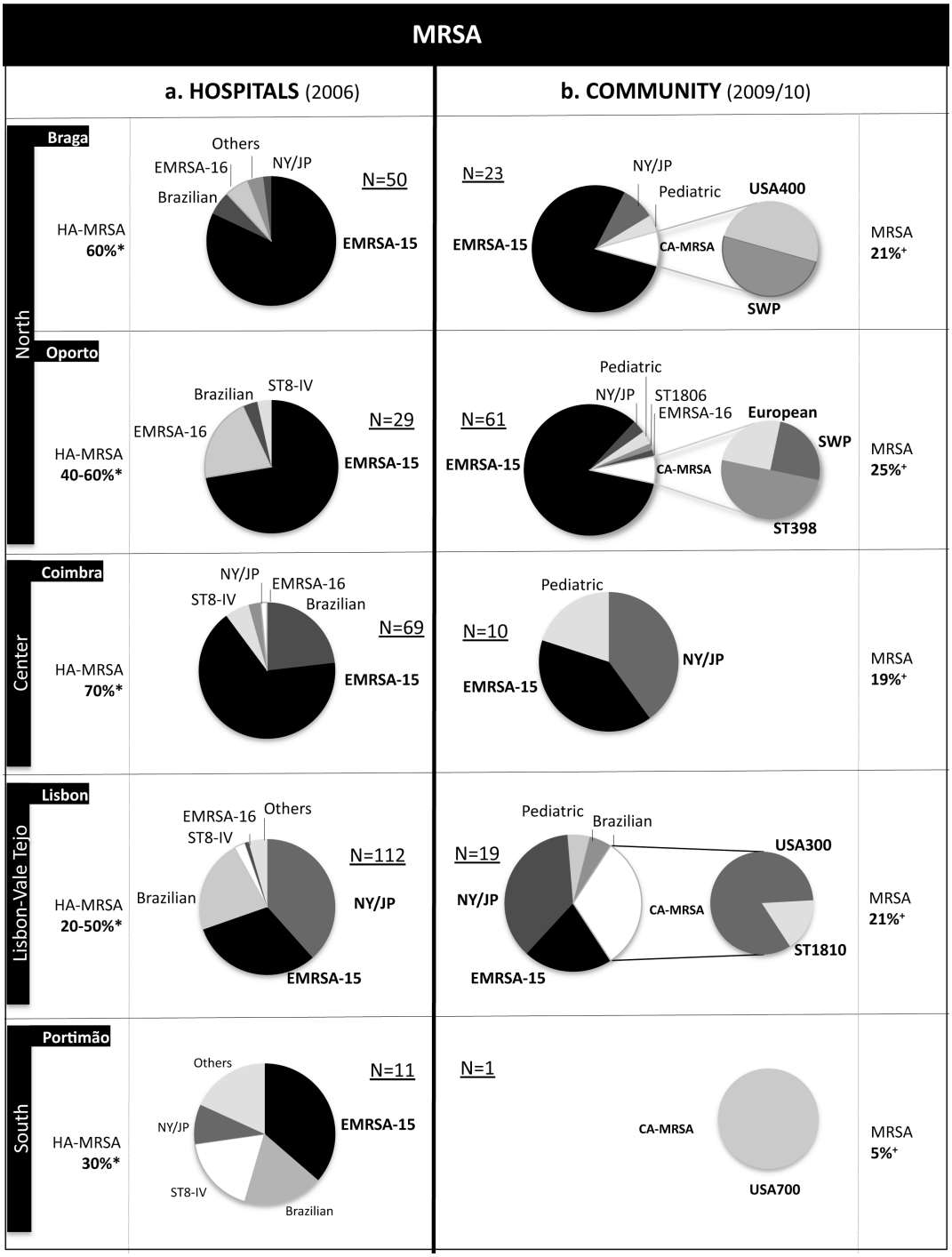


Figure. 1 Geographic distribution and prevalence of the representative community-acquired (CA) and hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones found in this study (2009/10) in comparison with HA-MRSA clones found in hospitals in 2006 (in the same geographic area) (8). *MRSA prevalence in the hospitals in 2009 (data provided); +MRSA prevalence in the community in 2009/10 (our study).

Population structure of MSSA

The MSSA population was represented by 413 isolates, and as expected showed a higher genetic diversity when compared to MRSA [SID=0.988 (0.984-0.993), SID=0.255 (0.174-0.335)]. A high number of *spa* types (76) and STs (58), represented by 29 CC, were detected. PVL was predominantly found in MSSA backgrounds (70.6%, n=12), namely, in isolates belonging to ST121 (n=6), ST8 (n=1), ST1 (n=1), ST2290 (n=1), ST36 (n=2), and ST5 (n=1).

The 413 MSSA isolates were divided into 210 isolates related to CA backgrounds and 203 isolates related to HA backgrounds.

Almost half of the MSSA isolates belonging to CA genetic backgrounds (46.7%, 98 out of 210 isolates) were related to epidemic clones, namely, USA700 (ST72-t126, t148) (30.3%, n=30), USA400 [ST1-t127, PVL (1)] (26.3%, n=26), USA300 [ST8-t008, t024, t064, PVL (1)] (18.2%, n=18), ST398 (ST398-t571, t1451, t034, t9865) (14.1%, n=14), SWP (ST30-t012, t021) (8.1%, n=8), and Taiwan (ST59-t216, t316) (3%, n=3). The remaining 112 isolates (53.3%) were represented by a wide diversity of STs (51) and spa types (86), including ST121, ST15, and ST25 clones that, nowadays, are considered epidemic.

A great proportion of the MSSA isolates that belonged to the HA genetic backgrounds (92.6%, 188 out of 203) were related to HA-MRSA epidemic clones and contained all the molecular features of the epidemic clones, except for the presence of SCC*mec*: EMRSA-16 (ST36, t018) (38.4%, n=71), NY/J (ST5, t002, t005, t071) (27.6%, n=54), Berlin (ST45, t015, t230, t050, t10942) (15.1%, n=28), EMRSA-15 (ST22, t005, t310) (9.7%, n=18), and Pediatric (ST5, t045) (9.2%, n=17).

The remaining 15 isolates belonged to HA genetic backgrounds, but the *spa* types found were not characteristic of epidemic clones, namely, 12 isolates belonging to ST45 (t1523, t1826, t2429, t330, t350, t550, t576), two isolates with ST2285 (SLV of ST45) (t1268), and one isolate with ST22 (t819).

Origin of contemporary MRSA in the community

Evidence for the emergence from an established MSSA population

In order to understand the possible origin of the MRSA clones found in the community in Portugal, the MRSA and MSSA populations were analyzed based on a comparison of the genetic backgrounds. The application of the goeBURST algorithm to MLST data showed that both MRSA lineages associated to the hospital (ST5, ST22, ST36, SLV ST239) and lineages associated to the

community (ST8, ST1, ST72, ST30, ST398) were related to the MSSA genetic backgrounds also established in our country, in the same time period (Figure 2).

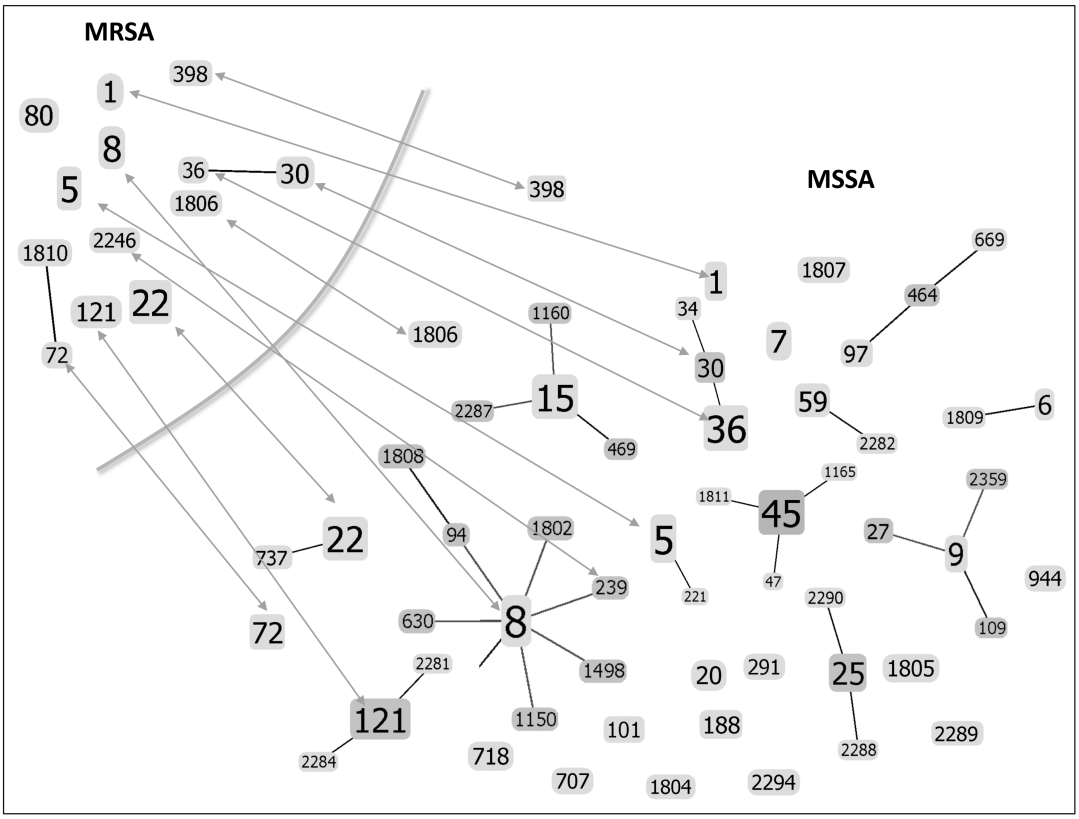


Figure 2. Analysis of the relationship between the MRSA and methicillin-susceptible *S. aureus* (MSSA) populations using multilocus sequence typing (MLST) data (goeBURST). The arrows indicate the sequence type (ST) present in both MRSA and MSSA populations. The analysis was performed with 527 isolates, where, for 155, the ST was determined and, for 372, the STwas inferred [pulsed-field gel electrophoresis (PFGE) and *spa* types].

The only exception was ST80, which we found only as MRSA. To better understand the degree of the relationship between MSSA and MRSA isolates, BURP analysis based on the spa types was performed. This analysis showed that MRSA and MSSA isolates belonging to ST5 (CC-t002, t179, t311), ST8 (CC-t008), ST30 (CC-t012), and ST398 (CC-t1451) shared the same or related *spa* types (Figure 3).

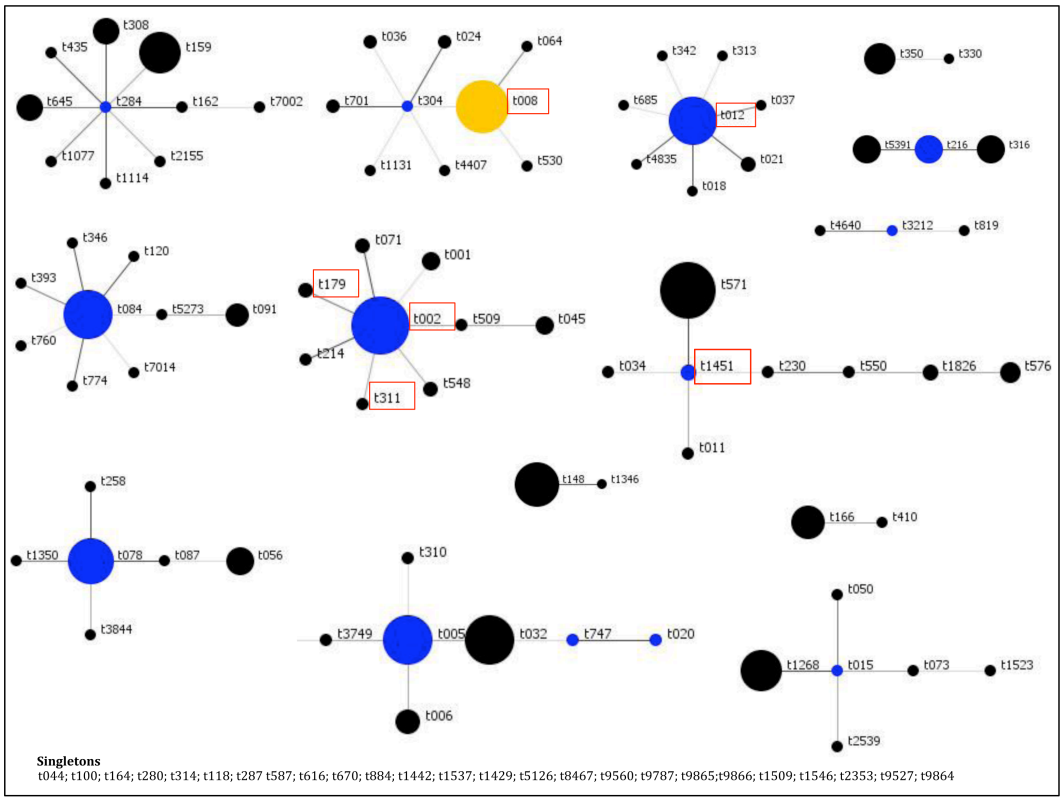


Figure 3. Analysis of the relationship between the MRSA and MSSA populations using spa type data (BURP). The highlighted numbers indicate related *spa* types in the MRSA and MSSA populations. The analysis was performed with 224 spa-typed isolates

For isolates for which the *spa* type was not determined, the relatedness was confirmed based on PFGE pattern comparison. This analysis showed that a set of 126 MRSA and MSSA isolates representative of each genetic background identified in our study were congruently clustered together and with more than 80% similarity with the reference CA- and HA-MRSA epidemic control strains, suggesting that they are related.

Evidence for MRSA dissemination from the hospital to the community

Interestingly, the distribution of MRSA clonal types by geographic region found in this study in the community was very similar to the MRSA distribution inside the Hospitals described in 2006 by Aires-de-Sousa (8) (Figure 1, a.). In Braga and Oporto hospitals, in 2006, the most predominant HA-MRSA clone was EMRSA-15 (82% and 72.4%, respectively) and we observed the same scenario in the community in the same region in 2009/10. While moving from the north to the south

of Portugal, the predominance of EMRSA-15 in the hospitals gradually decreased to 66.7% in Coimbra and 31.3% in Lisbon. The same north to south decreasing trend of EMRSA-15 frequency was observed in the community in this study. Additionally, in Lisbon, in both community and hospital settings, the NY/JP clone was more commonly represented than in other regions. The only exception to this parallelism between the population structure in the hospital and the community was observed in the Algarve region, where no HA clones were found in the community. Altogether, these observations suggest that most of the MRSA found in the community in Portugal came from hospitals.

DISCUSSION

During the last 20 years, we have been attending to an increase in the number of infection episodes in healthy individuals in the community. Despite the global concern about the burden of disease caused by CA-MRSA, in Portugal, only isolated studies describing MRSA with typical CA backgrounds were reported (17, 55, 63, 66, 71), and no structured surveillance was ever conducted. In this study, we report, for the first time, the prevalence, population structure, and epidemiology of MRSA that are causing infections in the community in Portugal.

We found a very high prevalence of MRSA (21.6%) circulating in the community in the country. This observation contrasts sharply with previous studies that reported much lower frequencies of CA-MRSA, both in colonization in children (<1%), outpatients (5.1%), and healthcare workers (4.8%), and in skin and soft tissue infections (SSTIs) (7.9%) (10, 17, 66, 71). The high rate of MRSA in the community was unexpected, and, to our knowledge, in Europe, only in Greece were such high frequencies seen (30%) (75). Other countries reporting in the last several years considerably high rates of MRSA in the community include France (18%), Spain (13%), and Italy (6.4%) (19, 45, 72). However, the great majority of MRSA found in the community belonged to clones typically found in the hospitals, in particular, the EMRSA-15 clone (77.2%). Additionally, the population structure of MRSA in the community in each geographic region appears to mimic that observed in hospitals. These data strongly suggest that HA-MRSA clones are escaping from hospitals into the community. Although not frequent, the phenomenon of dissemination of hospital MRSA in the community was previously reported in other European countries. In particular, in France, it was observed that the Lyon clone, the main clone found in the hospitals, corresponded to 80.6% of MRSA isolates found in the community (46) and the ST125-IV, the most widespread clone in the hospitals in Spain, corresponded to 58.6% of MRSA isolates found in the community (27). Moreover, dispersed reports of EMRSA-15 causing infection in the community were also described in Italy and England (31, 45).

The reasons lying behind the dissemination of HAMRSA from the hospital into the community are not clear. The fact that both Portugal and France have high frequencies of MRSA in hospitals might contribute to the invasion of the community with HA-MRSA, as previously observed for penicillin-resistant isolates in the 1950s. On the other hand, both the Lyon and the EMRSA-15 clones carry the smallest SCC*mec* cassette (SCC*mec* IV) and contain a few antimicrobial resistance

determinants only. Since the presence of SCC*mec* IV does not cause a significant fitness cost for the bacteria, EMRSA-15 seems to have high survival capacity (replicate, establish, and spread) in the community environment. Moreover, the EMRSA-15 clone has been reported to be able to accumulate typical CA-MRSA genetic features, namely, the presence of PVL, ACME, and a high expression of virulence genes, which might have increased its capacity to adapt to the community environment (13, 68).

It is widely accepted that the EMRSA-15 clone emerged in the English Midlands in the 1980s, and that, afterwards, has spread within and between the healthcare institutions, becoming endemic and is, nowadays, the most dominant clone in UK hospitals (36, 37, 64). Subsequently, global dissemination of the EMRSA-15 clone was observed to other geographic regions, including Portugal, Germany, the Netherlands, Denmark, Ireland, Sweden, the Czech Republic, Spain, Singapore, Australia, and New Zealand (8, 9, 26, 28, 33, 49, 61, 62, 78). In Portugal, in particular, EMRSA-15 clone was first described in 1997, in a Portuguese hospital located in Oporto (9), and, since then, has spread and established itself as the most predominant clone in virtually all major hospitals in this country (8). Moreover, the signs of spreading of EMRSA-15 into the community have already been found in the country, such as the heavy colonization of handrails on public buses (26% buses with 91% of the EMRSA-15 clone) and the colonization of dogs (30% of EMRSA-15 colonization) (16, 69).

The emergence of EMRSA-15 in the UK coincided with the introduction of ciprofloxacin (fluoroquinolone) into clinical practice in this country, which is believed to have triggered the development of ciprofloxacin resistance in strains of the EMRSA-15 clone (30). Horváth *et al.* recently described the impact of quinolone resistance on MRSA population dynamics in the Hungarian hospitals (32). In this study, the authors showed that ciprofloxacin resistance, conferred by two nonsynonymous mutations in *gyrA* and *grlA*, had a much lower fitness cost in the EMRSA-15 clone than in other HA-MRSA clones like NY/JP, South-German, Hungarian/Brazilian, or even the CA-MRSA ciprofloxacin derivatives mutants ST8- IV, ST80-IV, and ST30-IV. This “quinolone fitness competitive advantage” of EMRSA-15 may have contributed to the success of EMRSA-15 worldwide, especially in countries wherein the consumption of quinolones is high. In Portugal, in the last decade, quinolones have been extensively used both in the hospital (9) and in the community

(4). In fact, a recent national surveillance, performed in 2010 on the prevalence of nosocomial infection in Portugal, reported that quinolones (15%) was the second most often used class of antibiotics following penicillins and cephalosporins (46%) in the hospital setting (6), and similar rates of consumption have been observed in the community among ambulatory patients (4).

The actual national antimicrobial prescribing habits act as a selective pressure for keeping the EMRSA-15 clone in Portugal. Some studies have shown that the implementation of a fluoroquinolone control program can significantly decrease the MRSA rates in the hospital setting (41, 60). Similarly, the implementation of such a program might help to decrease the rates of MRSA in Portugal.

Additionally to the EMRSA-15 clone, also, the NY/JP clone, another clone associated to the hospital, was present in the community in Portugal. This clone, originally found in Japan in 1982 (34), was first described in 2005 in a Portuguese hospital located in Oporto (9) and was observed lately as the second most predominant clone in hospitals in the country (8). The presence of the NY/Japan clone outside the hospitals in Portugal, similarly to the EMRSA-15 clone, is probably related to the existent ciprofloxacin selective pressure (32), since most of the isolates from this clone show also resistance to ciprofloxacin.

The fraction of the MRSA found in the community that belongs to typical CA-MRSA epidemic clones is relatively low (2.5%). This seems to be a characteristic of several European countries such as Spain, Italy, Ireland, Switzerland, Germany, and England (40), and is in clear contrast to what is observed in the USA (54). Also, we found that MRSA belong to as many as six different CA-MRSA epidemic clones, which is in accordance to the type of population structure of CA-MRSA found in the 16 most populous European countries (65).

The few MRSA strains of CA-MRSA epidemic clones belong to USA300 (the most frequent), ST398, ST30, ST72, ST80, and ST1. The comparison of contemporary MSSA and MRSA populations allowed us to verify that, with the exception of ST80, all of these CA-MRSA genetic backgrounds exist in Portugal as MSSA. The results suggest that all of the MRSA isolates related to epidemic CA-MRSA clones could have emerged in Portugal by the acquisition of SCC*mec* by

established MSSA strains. However, this hypothesis still has to be proved by performing, for example, comparative genome analysis using whole genome sequencing (WGS).

Moreover, we cannot disregard the possibility that these CAMRSA clones were introduced in Portugal from abroad. In particular, the presence in Portugal of the prototype USA300 clone with multidrug resistance profile suggests that this clone could have been originated in the USA, where the predominance of multidrug-resistant USA300 has been extensively reported. Furthermore, one of the USA300 isolates contains type II ACME, which was only described in USA300 isolates originated in the USA (29), further reinforcing this hypothesis.

Moreover, the prototype ST398 (ST398-IVa, t001) was exclusively found in a particular rural area of Portugal among elderly women with SSTI. This livestock-associated MRSA genetic background had already been identified among pigs in Portugal (63), which probably results from the frequent pig commercial trade between Portugal, Spain, and Denmark, countries in which ST398 is frequent. Since the cases of infection with ST398 isolates were only found in this rural area, where pig farms are frequent, it is tempting to speculate that they could have resulted from the contact with pigs.

Some of the limitations of our study are in regard to the geographic area that was covered. Most of the hospitals included in this study were located in urban regions of the north and center of Portugal, whereas the south of Portugal and the rural areas were less represented. Although the study covers the great majority of the Portuguese population that lives mainly in the urban regions of the north and center of Portugal, important information may have been missed. Another limitation associated to the study was the lack of information in some of the questionnaires, which may imply that some of the isolates included in the study were collected from individuals that could have had risk factors for hospital contact. Also, the inclusion of additional questions in the questionnaire regarding travel and individuals' nationalities could have helped in the clarification of the origin of CA-MRSA epidemic clones found in Portugal. Irrespective of their origin, the high frequencies of MRSA in the community found in Portugal is worrisome and should be seen as a warning to the public health providers. Unless strict infection control measures and new antimicrobial prescription habits are adopted, the spreading of MRSA from the hospital into the community will continue. The

continuation of this type of surveillance study is, though, crucial in order to be aware of the standpoint of MRSA burden in Portugal and take appropriate action, particularly in prevention.

ACKNOWLEDGMENTS

We thank the Portuguese healthcare institutions that participated in this project (CA-MRSA/MSSA working group) and to all healthcare workers for their collaboration in the isolation of *S. aureus* isolates and completion of questionnaires.

The experimental work was done by A. Tavares, with the exception of the molecular characterization of 100 out of 527 *S. aureus*, which were performed by J. Rolo, C. Coelho, O. Bouchami and S. Ravaioli.

FUNDING

This study was funded by project Ref. P-99911 from Fundação Calouste Gulbenkian and additionally by FCT through grant Ref. Pest-OE/EQB/LAO004/2011, Portugal. A. Tavares was supported by grant SFRH/BD/44220/2008 from Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

REFERENCES

1. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement M100-S19. CLSI, Wayne, PA, USA, 2009).
2. The European Commission of the European Communities (2000) Commission decision of 22 December 1999 on the communicable diseases to be progressively covered by the community network under decision number 2119/98/EC of the Parliament and of the Council. Official J Eur Communities L 28/50. .
3. The European Parliament, the Council of the EU (1998) Decision number 2119/ 98/EC of the European Parliament and of the Council of 24 September 1998: setting up a network for the epidemiological surveillance and control of communicable diseases in the community. Official J Eur Communities L268/1.
4. European Surveillance of Antimicrobial Consumption. ESAC YearBook 2008 - ESAC Document prepared by the ESAC Management Team, the ESAC Scientific Advisory Board and the ESAC National Networks. University of Antwerp. 2010. www.esac.ua.ac.be.
5. 1999. From the Centers for Disease Control and Prevention. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*--Minnesota and North Dakota, 1997-1999. JAMA **282:**1123-5.
6. Relatório Inquérito de Prevalência de Infeção 2010. Programa Nacional de Prevenção e Controlo da Infeção Associada aos Cuidados de Saúde. Departamento da Qualidade na Saúde. Direcção Geral da Saúde. http://www.dgs.pt/.
7. **Aires de Sousa, M., C. Bartzavali, I. Spiliopoulou, I. S. Sanches, M. I. Crisostomo, 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.
8. **Aires-de-Sousa, M., B. Correia, and H. de Lencastre.** 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:**2912-7.
9. **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.
10. **Amorim, M. L., C. Vasconcelos, D. C. Oliveira, A. Azevedo, E. Calado, N. A. Faria, M. Pereira, A. P. Castro, A. Moreira, E. Aires, J. M. Cabeda, M. H. Ramos, J. M. Amorim, and H. de Lencastre.** 2009. Epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) nasal colonization among patients and healthcare workers in a Portuguese hospital: a pre-intervention study toward the control of MRSA. Microb Drug Resist **15:**19-26.
11. **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.
12. **Bens, C. C., A. Voss, and C. H. Klaassen.** 2006. Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis. J Clin Microbiol **44:**1875-6.
13. **Burlak, C., C. H. Hammer, M. A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo.** 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. Cell Microbiol **9:**1172-90.
14. **Chua, K., F. Laurent, G. Coombs, M. L. Grayson, and B. P. Howden.** 2011. Antimicrobial resistance: Not community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)! A clinician's guide to community MRSA - its evolving antimicrobial resistance and implications for therapy. Clin Infect Dis **52:**99-114.

15. **Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J. H. Song, P. T. Tassios, and P. Villari.** 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.
16. **Coelho, C., C. Torres, H. Radhouani, L. Pinto, C. Lozano, E. Gomez-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.
17. **Conceicao, T., M. Aires-de-Sousa, N. Pona, M. J. Brito, C. Barradas, R. Coelho, T. Sardinha, L. Sancho, G. de Sousa, M. do Ceu Machado, and H. de Lencastre.** 2010. High prevalence of ST121 in community-associated methicillin-susceptible *Staphylococcus aureus* lineages responsible for skin and soft tissue infections in Portuguese children. Eur J Clin Microbiol Infect Dis.
18. **Coombs, G. W., G. R. Nimmo, J. M. Bell, F. Huygens, F. G. O'Brien, M. J. Malkowski, J. C. Pearson, A. J. Stephens, and P. M. Giffard.** 2004. Genetic diversity among community methicillin-resistant *Staphylococcus aureus* strains causing outpatient infections in Australia. J Clin Microbiol **42:**4735-43.
19. **Daskalaki, M., P. Rojo, M. Marin-Ferrer, M. Barrios, J. R. Otero, and F. Chaves.** 2010. Panton-Valentine leukocidin-positive *Staphylococcus aureus* skin and soft tissue infections among children in an emergency department in Madrid, Spain. Clin Microbiol Infect **16:**74-7.
20. **David, M. Z., and R. S. Daum.** Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev **23:**616-87.
21. **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.
22. **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.
23. **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.
24. **Fan, J., M. Shu, G. Zhang, W. Zhou, Y. Jiang, Y. Zhu, G. Chen, S. J. Peacock, C. Wan, W. Pan, and E. J. Feil.** 2009. Biogeography and virulence of *Staphylococcus aureus*. PLoS One **4:**e6216.
25. **Faria, N. A., J. A. Carrico, 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.
26. **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.
27. **Gonzalez-Dominguez, M., C. Seral, Y. Saenz, S. Salvo, M. J. Gude, N. Porres-Osante, C. Torres, and F. J. Castillo.** 2012. Epidemiological features, resistance genes, and clones among community-onset methicillin-resistant *Staphylococcus aureus* (CO-MRSA) isolates detected in northern Spain. Int J Med Microbiol **302:**320-6.
28. **Gosbell, I. B., T. Barbagiannakos, S. A. Neville, J. L. Mercer, A. M. Vickery, F. G. O'Brien, G. W. Coombs, M. J. Malkowski, and J. C. Pearson.** 2006. Non-multiresistant methicillin-resistant *Staphylococcus aureus* bacteraemia in Sydney, Australia: emergence of EMRSA-15, Oceania, Queensland and Western Australian MRSA strains. Pathology **38:**239-44.

29. **Hedlund, L., B. Hellmark, and B. Soderquist.** 2012. Presence of arginine catabolic mobile element among community-acquired meticillin-resistant *Staphylococcus aureus* is linked to a specific genetic background. APMIS.
30. **Holden, M. T., L. Y. Hsu, K. Kurt, L. A. Weinert, A. E. Mather, S. R. Harris, B. Strommenger, F. Layer, W. Witte, H. de Lencastre, R. Skov, H. Westh, H. Zemlickova, G. Coombs, A. M. Kearns, R. L. Hill, J. Edgeworth, I. Gould, V. Gant, J. Cooke, G. F. Edwards, P. R. McAdam, K. E. Templeton, A. McCann, Z. Zhou, S. Castillo-Ramirez, E. J. Feil, L. O. Hudson, M. C. Enright, F. Balloux, D. M. Aanensen, B. G. Spratt, J. R. Fitzgerald, J. Parkhill, M. Achtman, S. D. Bentley, and U. Nubel.** 2013. A genomic portrait of the emergence, evolution and global spread of a methicillin resistant *Staphylococcus aureus* pandemic. Genome Res.
31. **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.
32. **Horvath, A., O. Dobay, S. Kardos, A. Ghidan, A. Toth, J. Paszti, E. Ungvari, P. Horvath, K. Nagy, S. Zissman, and M. Fuzi.** 2012. Varying fitness cost associated with resistance to fluoroquinolones governs clonal dynamic of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis **31:**2029-36.
33. **Hsu, L. Y., T. H. Koh, K. Singh, M. L. Kang, A. Kurup, and B. H. Tan.** 2005. Dissemination of multisusceptible methicillin-resistant *Staphylococcus aureus* in Singapore. J Clin Microbiol **43:**2923-5.
34. **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.
35. **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.
36. **Johnson, A. P., H. M. Aucken, S. Cavendish, M. Ganner, M. C. Wale, M. Warner, D. M. Livermore, and B. D. Cookson.** 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.
37. **Johnson, A. P., A. Pearson, and G. Duckworth.** 2005. Surveillance and epidemiology of MRSA bacteraemia in the UK. J Antimicrob Chemother **56:**455-62.
38. **Khanna, T., R. Friendship, C. Dewey, and J. S. Weese.** 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet Microbiol **128:**298-303.
39. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin.** 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA **298:**1763-71.
40. **Kock, R., K. Becker, B. Cookson, J. E. van Gemert-Pijnen, S. Harbarth, J. Kluytmans, M. Mielke, G. Peters, R. L. Skov, M. J. Struelens, E. Tacconelli, A. Navarro Torne, W. Witte, and A. W. Friedrich.** 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. Euro Surveill **15:**19688.
41. **Lafaurie, M., R. Porcher, J. L. Donay, S. Touratier, and J. M. Molina.** 2012. Reduction of fluoroquinolone use is associated with a decrease in methicillin-resistant *Staphylococcus aureus* and fluoroquinolone-resistant *Pseudomonas aeruginosa* isolation rates: a 10 year study. J Antimicrob Chemother **67:**1010-5.
42. **Larsen, A. R., M. Stegger, S. Bocher, M. Sorum, D. L. Monnet, and R. L. Skov.** 2009. Emergence and characterization of community-associated methicillin-resistant Staphyloccocus aureus infections in Denmark, 1999 to 2006. J Clin Microbiol **47:**73-8.

43. **Lina, G., Y. Piemont, 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.
44. **Manzur, A., A. M. Dominguez, M. Pujol, M. P. Gonzalez, E. Limon, A. Hornero, R. Martin, F. Gudiol, and J. Ariza.** 2008. Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. Clin Microbiol Infect **14:**377-80.
45. **Marchese, A., L. Gualco, E. Maioli, and E. Debbia.** 2009. Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int J Antimicrob Agents **34:**424-8.
46. **Maugat, S., A. de Rougemont, H. Aubry-Damon, M. E. Reverdy, S. Georges, F. Vandenesch, J. Etienne, and B. Coignard.** 2009. Methicillin-resistant *Staphylococcus aureus* among a network of French private-sector community-based-medical laboratories. Med Mal Infect **39:**311-8.
47. **Mediavilla, J. R., L. Chen, B. Mathema, and B. N. Kreiswirth.** 2012. Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Curr Opin Microbiol **15:**588-95.
48. **Mellmann, A., T. Weniger, C. Berssenbrugge, J. Rothganger, 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.
49. **Melter, O., P. Urbaskova, V. Jakubu, B. Mackova, and H. Zemlickova.** 2006. Emergence of EMRSA-15 clone in hospitals throughout the Czech Republic. Euro Surveill **11:**E060803 6.
50. **Milheirico, 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.
51. **Milheirico, 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.
52. **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.
53. **Montgomery, C. P., S. Boyle-Vavra, P. V. Adem, J. C. Lee, A. N. Husain, J. Clasen, and R. S. Daum.** 2008. Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. J Infect Dis **198:**561-70.
54. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **355:**666-74.
55. **Nazareth, R., J. Goncalves-Pereira, A. Tavares, M. Miragaia, H. de Lencastre, J. Silvestre, P. Freitas, E. Goncalves, F. Martins, V. Mendes, C. Tapadinhas, and P. Povoa.** 2012. Community-associated methicillin-resistant *Staphylococcus aureus* infection in Portugal. Rev Port Pneumol **18:**34-8.
56. **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.
57. **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.
58. **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.

59. **Oliveira, D. C., C. Milheirico, S. Vinga, and H. de Lencastre.** 2006. Assessment of allelic variation in the *ccrAB* locus in methicillin-resistant *Staphylococcus aureus* clones. J Antimicrob Chemother **58:**23-30.
60. **Parienti, J. J., V. Cattoir, P. Thibon, G. Lebouvier, R. Verdon, C. Daubin, D. du Cheyron, R. Leclercq, and P. Charbonneau.** 2011. Hospital-wide modification of fluoroquinolone policy and meticillin-resistant *Staphylococcus aureus* rates: a 10-year interrupted time-series analysis. J Hosp Infect **78:**118-22.
61. **Pearman, J. W., G. W. Coombs, W. B. Grubb, and F. O'Brien.** 2001. A British epidemic strain of methicillin-resistant *Staphylococcus aureus* (UK EMRSA-15) in Western Australia. Med J Aust **174:**662.
62. **Perez-Roth, E., F. Lorenzo-Diaz, N. Batista, A. Moreno, and S. Mendez-Alvarez.** 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.
63. **Pomba, C., H. Hasman, L. M. Cavaco, J. D. da Fonseca, and F. M. Aarestrup.** 2009. First description of meticillin-resistant *Staphylococcus aureus* (MRSA) CC30 and CC398 from swine in Portugal. Int J Antimicrob Agents **34:**193-4.
64. **Richardson, J. F., and S. Reith.** 1993. Characterization of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. J Hosp Infect **25:**45-52.
65. **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.
66. **Sa-Leao, R., I. S. Sanches, I. Couto, C. R. Alves, and H. de Lencastre.** 2001. Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist **7:**237-45.
67. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, 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.
68. **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 ST22-MRSA-IV. Antimicrob Agents Chemother **55:**1896-905.
69. **Simoes, R. R., M. Aires-de-Sousa, T. Conceicao, 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.
70. **Simpson, E.** 1949. Measurement of diversity. Nature **163: 688**.
71. **Tavares, D. A., R. Sa-Leao, M. Miragaia, and H. de Lencastre.** 2010. Large screening of CA-MRSA among *Staphylococcus aureus* colonizing healthy young children living in two areas (urban and rural) of Portugal. BMC Infect Dis **10:**110.
72. **Thibaut, S., J. Caillon, C. Huart, G. Grandjean, P. Lombrail, G. Potel, and F. Ballereau.** 2010. Susceptibility to the main antibiotics of *Escherichia coli* and *Staphylococcus aureus* strains identified in community acquired infections in France (MedQual, 2004-2007). Med Mal Infect **40:**74-80.
73. **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.
74. **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. Paszti, 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.

75. **Vourli, S., H. Vagiakou, G. Ganteris, M. Orfanidou, M. Polemis, A. Vatopoulos, and H. Malamou-Ladas.** 2009. High rates of community-acquired, Panton-Valentine leukocidin (PVL)-positive methicillin-resistant *S. aureus* (MRSA) infections in adult outpatients in Greece. Euro Surveill **14**.
76. **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:**1510-4.
77. **Watkins, R. R., M. Z. David, and R. A. Salata.** 2012. Current concepts on the virulence mechanisms of meticillin-resistant *Staphylococcus aureus*. J Med Microbiol **61:**1179-93.
78. **Witte, W., M. Enright, F. J. Schmitz, C. Cuny, C. Braulke, and D. Heuck.** 2001. Characteristics of a new epidemic MRSA in Germany ancestral to United Kingdom EMRSA 15. Int J Med Microbiol **290:**677-82.
79. **Witte, W., B. Strommenger, C. Cuny, D. Heuck, and U. Nuebel.** 2007. Methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006. J Antimicrob Chemother **60:**1258-63.

SUPPLEMENTARY MATERIAL

Table S1. Distribution of demographic and clinical data among CA-MRSA and HA-MRSA clonal types.

	CA-MRSA background n=13 (%)	**HA-MRSA background** n=101 (%)	***p*-value**
Age group			
Pediatric (0-18 Years)	2 (15.4)	11 (10.9)	0.63
Adult (19-64 Years)	8 (61.5)	36 (35.6)	0.07
Elderly (>65 Years)	3 (23.1)	53 (52.6)	0.05
No data	-	1 (1)	-
Gender			
Male	4 (30.8)	55 (54.5)	0.11
Female	9 (69.2)	45 (44.6)	0.09
No data	-	1 (1)	-
Clinical relevance			
Infection	12 (92.3)	88 (87.1)	0.59
Colonization	-	12 (11.9)	0.19
No data	1 (7.7)	1 (1)	-
Clinical presentation			
Skin and Soft Tissue Infections	5 (38.5)	49 (48.5)	0.49
Pneumonia and respiratory infections	2 (15.4)	14 (13.9)	0.88
Bacteremia, septicemia, endocarditis	2 (15.4)	2 (2)	0.01
Urinary tract infections	-	3 (3)	0.53
Others	2 (15.4)	25 (24.8)	0.45
No data	2 (15.4)	8 (7.9)	-
Sample origin			
Medical appointment	2 (15.4)	31 (30.7)	0.25
Internment	3 (28.1)	27 (26.7)	0.78
Clinical laboratory	2 (15.4)	19 (18.8)	0.76
Emergency unit	6 (46.2)	22 (21.8)	0.05
Health care center	-	1 (1)	0.72
No data	-	1 (1)	-

CHAPTER III

Population structure of methicillin-susceptible *Staphylococcus aureus* (MSSA) in Portugal over a 19 years period (1992 to 2011)

THIS CHAPTER WAS PUBLISHED IN:

Tavares, A, Faria N. A., de Lencastre H., and M. Miragaia. 2014. Population structure of methicillin-susceptible *Staphylococcus aureus* (MSSA) in Portugal over a 19 years period (1992 to 2011). Eur J Clin Microbiol Infect Dis. 33(3):423-32.

ABSTRACT

Background

Despite their clinical relevance, few studies have addressed the epidemiology of methicillin-susceptible *S. aureus* (MSSA). In particular, it is not clear how MSSA population structure has evolved over time and how it might have been shaped by the emergence of MRSA in the community. In the present study we have evaluated the MSSA population structure overtime, its geographical distribution and relatedness with MRSA in Portugal.

Methods

A total of 465 MSSA from infection and colonization, collected over a 19 years period (1992-2011) in the North, Center and South of Portugal were analyzed. Isolates were characterized by *spa* typing and multilocus-sequence typing (MLST). Isolates with predominant *spa* types were characterized by pulsed-field gel electrophoresis (PFGE). Isolates relatedness was analyzed by goeBURST and BURP.

Results

The total of 172 *spa* types found among the 465 MSSA were grouped into 18 *spa*-CC (clonal complexes). Ten clonal-types were more prevalent (40%): one major clone (ST30-t012) was present in the entire study period and all over the country; the other nine were intermittently detected over time (ST5-t002, ST8-t008, ST15-t084, ST34-t166, ST72-t148, ST1-t127, ST7-t091, ST398-t571 and ST34-t136). Interestingly, three MSSA clonal types observed only after 1996 were closely related with CA-MRSA epidemic strains (ST8-t008, ST72-t148 and ST1-t127) found currently in Portugal.

Conclusion

The MSSA population in Portugal is genetically diverse; however some dominant clonal types have been established and widely disseminated for almost two decades. We identified MSSA isolates that were related with emergent CA-MRSA clones found in Portugal.

INTRODUCTION

The acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*), carrying the determinant of resistance to methicillin and to the entire class of beta-lactams (*mecA*) by methicillin-susceptible *S. aureus* (MSSA) was a hallmark in the history of *S. aureus* as a human pathogen. Nowadays, methicillin-resistant *S. aureus* (MRSA) are one of the most important nosocomial pathogens worldwide (31, 32).

According to EARS-Net report from 2011 (12), Portugal is the European country with the highest rate of MRSA in invasive disease (54.6%). The nosocomial MRSA population structure in Portugal has been extensively characterized and four successive clonal waves have been described (3). First in early 1992/93, the widespread Portuguese clone (ST239-III variant) was replaced by the Iberian clone (ST247-I), followed by the emergence in 1994/95 of the multiresistant Brazilian clone (ST239-IIIA), with rapid dissemination all over the country. Afterwards, there was a massive replacement of the Brazilian clone by the epidemic EMRSA-15 (ST22-IV), currently the most predominant clone in Portuguese Hospitals, accounting for 72% of all MRSA isolates (2, 3, 14). Nearly after the emergence of EMRSA-15, a variant of the New York/Japan (NY/JP) (ST105-II) appeared as the second most predominant clone in Portuguese hospitals. Recently, a high percentage of MRSA (21.6%) was found also in community in Portugal, where the EMRSA-15 or related clones were the predominant clones (77.2%), followed by NY/JP or related clones (14.9%) (30).

As the result of the complexity of the treatment and associated burden, MRSA have been given much more attention than MSSA. In fact, although MSSA have been also frequently associated to acute infections (10), their epidemiology is largely unexplored. The few studies available showed that MSSA have a higher degree of genetic diversity and a wider geographic distribution, in opposition to the clonality and geographic clustering found in MRSA (18, 20). Regarding the relatedness between MSSA and MRSA, results obtained so far indicated that MSSA and MRSA belonging to specific genetic backgrounds can co-exist in the same geographic location and time period, but the frequency of such phenomena varies with the geographic region analyzed (1, 10, 18, 20).

A previous study analyzing the population structure of MSSA in Portugal showed that ST30, ST34, ST5, and ST45 were the most predominant clonal types found between 1992 and 2001 in the community and hospital. Moreover, this same study showed that the genetic backgrounds of dominant nosocomial MRSA clones found over a 15 year-period (Iberian, Brazilian, and EMRSA-15) were scarcely or not found among MSSA. (1), suggesting that acquisition of SCC*mec* by resident epidemic MSSA backgrounds was not frequent in the country.

In the present study we aimed to further contribute to the knowledge of MSSA epidemiology by assessing MSSA population dynamics and geographical distribution over a 19 years period, in a country with a high MRSA prevalence. Moreover, we intended to understand how the emergence of CA-MRSA in Portugal might have shaped the MSSA population structure.

MATERIAL AND METHODS

Isolates collection

The 465 MSSA isolates from this study included 333 community-onset isolates, 123 hospital-associated isolates, and nine of unknown epidemiological origin; the isolates were recovered in Portugal between 1992 to 2011 from different populations.

Isolates were considered as community-onset (CO) when collected from outpatients and from individuals within 48 hours after hospital admission or after clinical symptoms developed, and as hospital-associated (HA) when collected from inpatients (≥48 hours of hospitalization or clinical symptoms developed).

The 333 CO-MSSA isolates were recovered in different studies: 1) nasopharyngeal colonization from children (0-5 years old) attending 16 day care centers (DCC) located in Lisbon (1996, 1997) (n=60) (28); 2) nasal colonization of military draftees (DFT) originally from Lisbon and Oporto (1996/97) (n=12) (28); and 3) infection and colonization from individuals attending 12 hospitals (within 48 hours of hospitalization), a clinical laboratory and a health care centre, covering the North (n=141), Center (n=28), Lisbon (n=37), and South (n=11) of Portugal (2009/10) (30) and 4) 44 isolates that were recovered from invasive disease of individuals where symptoms were developed within the 48 hours after Hospital admission in nine Portuguese Hospitals (North, n=20; Center, n=2; Lisbon, n=15; South, n=7) (2011) (Faria *et al.* in preparation).

The 123 HA-MSSA isolates were collected in ten hospitals: 1) 89 isolates from infection and colonization from two hospitals located in the North of Portugal (Oporto) (1992/1993, 2001) (n=85) and Lisbon (1995/96) (n=4) (1) and 2) 34 isolates from patients with invasive disease developed more than 48 hours after admission in ten Portuguese Hospitals (North, n=7; Center, n=7; Lisbon, n=17; South, n=3) (2011) (Faria *et al.* in preparation). Moreover, nine isolates where included, recovered in a Hospital located in Lisbon (2011), where no epidemiological context information was available (16).

For each isolate, information was collected regarding patient demographic information (age and gender) and general clinical and microbiological data, namely biological specimens, clinical relevance, clinical diagnosis and information on susceptibility to oxacillin (OXA) (n=378) or cefoxitin (FOX) (n=87).

Isolates demographics

Among the 465 isolates included in this study, about half (53.3%, n=248 isolates) were collected from males and 35.5 % (n=165) from females. For 11.2% of isolates (n=52), no data was available concerning the gender. The isolates were recovered mainly from adults (18-64 years) (43.4%, n=202), but also from the elderly (>65)(32.7%, n=152) and children (0–18) (21.7%, n=101). No information was available for 10 isolates (2.2%).

Isolates clinical relevance and sources

More than half of the isolates (61%, n=285) were from infection and 27% (n=124) from colonization. For 12% of the isolates (n=56) no data was available regarding their clinical relevance. A considerable part of community-onset isolates were from infections (69.4%, n=231), predominantly skin and soft tissue infections (43.1%, n=100), but also blood stream infections (22.8%, n=53), pneumonia and respiratory infections (9.5%, n=22) and other types of infections (20.7%, n=48). For eight isolates (3.4%), no data was available. Considering the nosocomial isolates, no information was available regarding clinical relevance for more than one third of isolates (35%, n=43). The remaining isolates were from infections (37%, n=45), mainly from blood stream infections (77.8%, n=35), and from colonization (28%, n= 35).

Molecular characterization

Detection of *mecA*

The presence of *mecA* was determined by PCR amplification (22, 26). Isolates were considered to be MSSA when *mecA* was absent, irrespective of their susceptibility to oxacillin and cefoxitin.

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

For all isolates, the *spa* type was determined as previously described (29). The *spa* types were assigned using Ridom-Staph software updated with RIDOM database (http://spaserver.ridom.de). Using the integrated BURP (Based Upon Repeat Patterns) algorithm, *spa* types were clustered into *spa* clonal complexes (*spa*-CC) according to the following settings: *spa* types clustered if cost were less than 4, and repeats were excluded if shorter than five (23).

Pulsed-field gel electrophoresis (PFGE)

For 77% (142 out of 185) isolates representative of the 10 most predominant *spa* types found (see Figure 1), PFGE was performed as previously described (8). The resultant SmaI band pattern profiles of isolates were compared with PFGE of reference CA and HA-MRSA epidemic control strains by using the BioNumerics software version 6.6 (Applied Maths, Saint-Martens-Latem, Belgium) with previously defined settings (optimization: 0.5%, tolerance: 1.3%, cut-off: 80%) (15). The isolates resistant to digestion with SmaI were classified as putative livestock-associated clone ST398, since this characteristic is specific of this clone (5).

Multilocus sequence typing (MLST)

The sequence type (ST) was determined for 182 isolates, including representative isolates of the most predominant *spa* types and less disseminated *spa* types. The MLST was performed as previously described (13). STs were attributed according to the MLST database (http://www.mlst.net/). For the remaining 283 isolates, the STs were inferred based on PFGE similarity with reference epidemic clones, *spa* server data (http://spaserver.ridom.de) or data obtained from the literature. To evaluate population structure and patterns of evolution, the eBURST algorithm (17) was used in order to assign MLST clonal complexes (MLST-CC) (http://saureus.mlst.net/sql/burst_all.asp, eBURST v.3). Additionally, MLST clonal complexes were restricted to single and double locus variants (SLV and DLV, respectively) from the respective founder or sub founders, within each clonal group. The only exception was CC6, which despite having as founder ST6, is a DLV from ST5. In this case and since ST6 has its own SLV descendants, and is characterized by a different *spa* type, its was considered as an independent CC. This analysis was performed on June 20, 2013.

Statistical analysis

Significant statistical differences were determined using the $\chi 2$. A *p*-value ≤ 0.05 was considered to be significantly different.

The degree of genetic diversity was calculated using Simpson's Index of Diversity (SID) with 95% confidence interval (http://darwin.phyloviz.net/). The SID was estimated using a *spa* type as a unit.

RESULTS

MSSA population structure

Among the 465 MSSA isolates, a total of 172 *spa* types and 69 STs were identified. Population structure analysis based on eBURST showed that the 69 STs were clustered in 24 MLST-CC and 8 singletons (see Table 1) The most predominant MLST-CC were CC30 (22.4%, n=104) including ST30 and ST34, followed by CC5 (10.6%, n=49), CC8 (8.6%, n=40), CC15 (8.6%, n=38), CC45 (8.6%, n=40), CC121 (5.8%, n=27), CC72 (5.8%, n=26), CC398 (3.9%, n=18), CC1 (3.6%, n=17), CC25 (3.6%, n=17), CC9 (3%, n=14), CC22 (2.6%, n=12), and CC7 (2.4%, n=11). The remaining eleven CCs and 8 singletons included approximately 11% of the isolates (10.9%, n=51).

The most dominant STs were ST30 (12.5%, n=58), ST5 (10.3%, n=48), ST34 (9.9%, n=46), ST15 (7.5%, n=35), ST8 (7.3%, n=34), ST45 (6.5%, n=30), ST121 (6.0%, n=28) and in a less extent ST72 (4.9%, n=23), ST398 (3.7%, n=17), ST25 (3.4%, n=16), ST1 (3.4%, n=16), ST7 (2.4%, n=11) and ST9 (2.2%, n=10). The remaining 20% of isolates (n=93) included 56 different STs.

Population structure analysis based on BURP showed that the 172 *spa* types were clustered in 18 *spa*-CCs, including three *spa*-CCs with no founder, and 19 singletons (See Figure 1). A total of 15 *spa* types were excluded from the analysis because the *spa* region was less than four repeats in length. The most predominant *spa*-CC found was *spa*-CC012, representing 12.9% of the isolates (n=60), followed by *spa*-CC002 (10.3%, n=48), *spa*-CC015 (9.9%, n=46) and *spa*-CC084 (9.9%, n=46), *spa*-CC008 (9.0%, n=42), *spa*-CC166 (8.4%, n=39), and *spa*-CC284 (5.0%, n=23). The remaining *spa*-CC contained less than 5% of the isolates each (See Table 1 and Figure 1). These *spa*-CCs were associated with genetic lineages commonly found among epidemic MRSA clones but also less disseminated clones, e.g. *spa*-CC012-ST30, *spa*-CC002–ST5, *spa*-CC008-ST8, *spa*-CC166-ST34, *spa*-CC005-ST22, *spa*-CC015/065-ST45, *spa*-CC015-ST398, *spa*-CC1346-ST72, *spa*-CC127-ST1, *spa*-CC216-ST59, *spa*-CC284–ST121, *spa*-CC078/280-ST25, *spa*-CC084-ST15/ST7 and *spa*-CC359-ST97 (See Table 1).

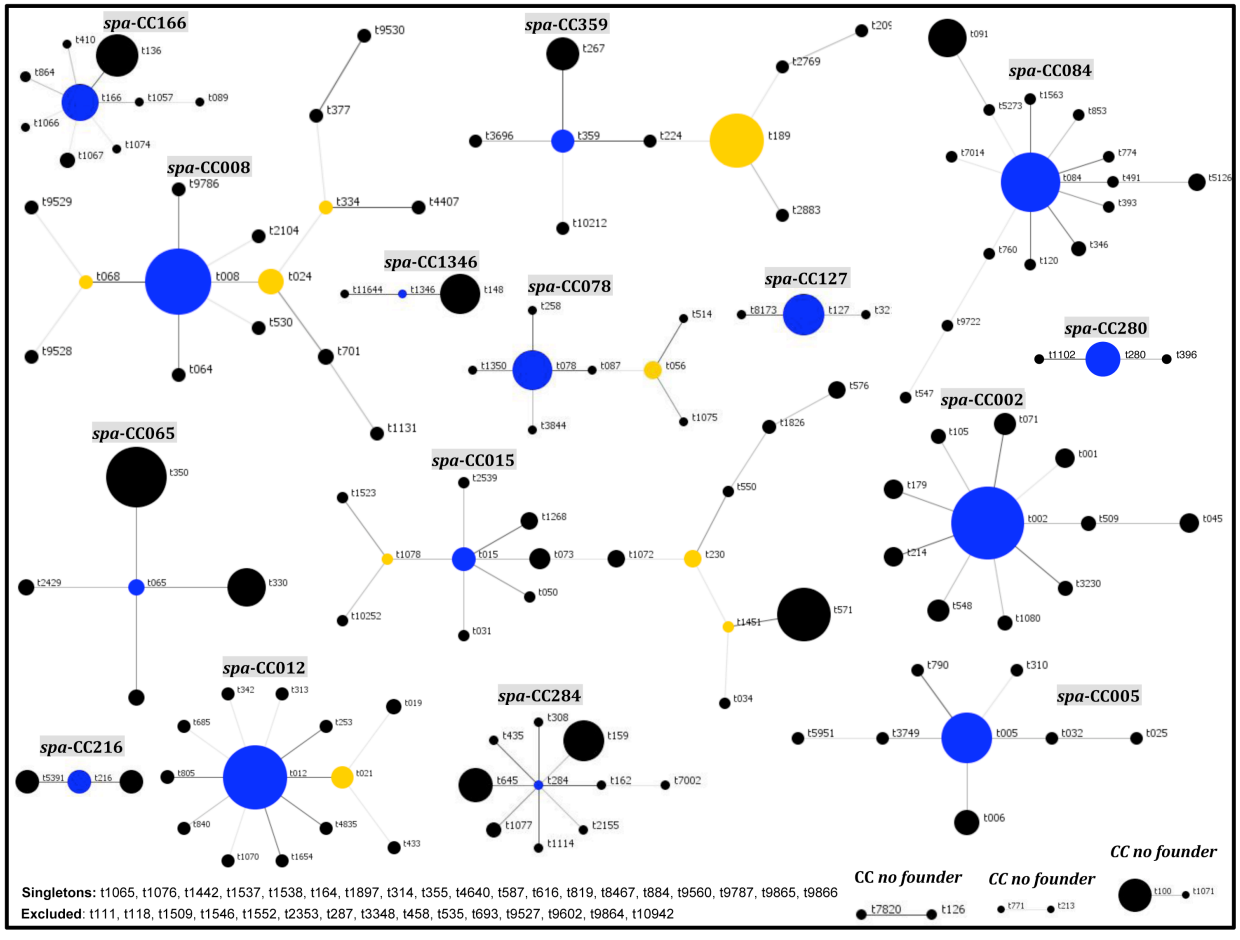


Figure 1. Analysis of *spa* typing data using BURP algorithm. Each number indicate a *spa* type, related *spa* types are linked with lines and resultant *spa* clonal complexes (*spa*-CC) delimited by line boxes. The predicted founder of each *spa*-CC is indicated in blue and named, and sub founders inside the *spa*-CC are indicated in yellow, respectively. The size of the circles is proportional to the frequency of the *spa* type.

Table 1. Distribution of MSSA isolates by *spa*-CC and the three most predominant *spa* types.

spa-CC[1]	No. (%) isolates	No. *spa* types	SID 95% CI[2]	Most predominant *spa* types (No. isolates)				MLST	MLST-CC[3]
				First	Second	Third	Others		
CC012	**60** (12.9)	13	0.585 (0.442-0.727)	**t012 (38)**	t021 (8)	t805 (2)	t019 (3); t1070 (1); t253 (1); t313 (1); t342 (1); t4835 (1); t685 (1); t840 (1); t1654 (1); t433 (1)	ST30 (57); ST2292 (1); ST1807 (1); ST1805 (1)	CC30 (58); CC97 (1); Singleton (1)
CC002	**48** (10.3)	11	0.805 (0.728-0.881)	**t002 (23)**	t045 (4); t071 (4); t548 (4)	t001 (3); t214 (3); t179 (3)	t3230 (1); t509 (1); t105 (1); t1080 (1)	ST5 (46); ST221(1); ST1804 (1)	CC5 (47); Singleton (1)
CC015	**46** (9.9)	17	0.881 (0.832-0.930)	**t571 (14)**	t015 (5)	t073 (4)	t1072 (3); t1268 (3); t230 (3); t576 (3); t1826 (2); t031 (1); t550 (1); t034 (1); t050 (1); t1078 (1); t1451 (1); t1523 (1); t2539 (1); t10252 (1)	ST45 (20); ST398 (16); ST2285 (3); ST47 (3); ST1811; (1); ST1165 (1); ST508 (1); ST1 (1)	CC45 (29); CC398 (16); CC1 (1)
CC084	**46** (9.9)	15	0.819 (0.760-0.877)	**t084 (19)**	**t091 (11)**	t5126 (3)	t346 (2); t120 (1); t393 (1); t5273 (1); t547 (1); t7014 (1); t760 (1); t774 (1); t853 (1); t9722 (1); t1563 (1); t491 (1)	ST15 (32); ST7 (11); ST469 (1); ST2287 (1); ST1160 (1)	CC15 (35); CC7 (11)
CC008	**42** (9.0)	15	0.774 (0.700-0.848)	**t008 (22)**	t024 (6)	t701 (2)	t064 (1); t068 (1); t1131 (1); t530 (1); t9528 (1); t9529 (1); t9530 (1); t9786 (1); t2104 (1); t334 (1); t377 (1); t4407 (1)	ST8 (34); ST6 (3); ST94 (1); ST1803 (1); ST1498 (1); ST1809 (1); ST630 (1)	CC8 (38); CC6 (4)
CC166	**39** (8.4)	9	0.774 (0.718-0.830)	**t136 (15)**	**t166 (13)**	t1067 (4)	t864 (2); t089 (1); t1057 (1); t1066 (1); t1074 (1); t410 (1)	ST34 (37); ST2291 (1); ST2289 (1)	CC30 (37); CC72 (1); CC10 (1)
CC284	**23** (5.0)	10	0.602 (0.465-0.740)	t159 (7)	t645 (6)	t1077 (2)	t2155 (2); t1114 (1); t162 (1); t284 (1); t308 (1); t435 (1); t7002 (1)	ST121 (21); ST2281 (1); ST1841 (1)	CC121 (23)
CC1346	**21** (4.5)	3	0.189 (1.000-0.404)	**t148 (18)**		-	t1346 (2); t11644 (1)	ST72 (19); ST615 (2)	CC72 (21)
CC078	**17** (3.7)	8	0.472 (0.323-0.622)	t078 (8)	t056 (3)	-	t087 (1); t1075 (1); t1350 (1); t258 (1); t3844 (1); t514 (1)	ST25 (10); ST101 (3); ST106 (2); ST6 (1); ST2288 (1)	CC25 (11); CC101 (5); CC6 (1)
CC359	**16** (3.4)	9	0.458 (0.300-0.615)	t189 (5)	t267 (3)	t359 (2)	t209 (1); t224 (1); t2769 (1); t2883 (1); t3696 (1); t10212 (1)	ST97 (6); ST188 (7); ST464 (1); ST2293 (1); ST109 (1)	CC97 (7); CC188 (7); CC8 (1); CC9 (1)
CC127	**14** (3.0)	3	0.378 (0.247-0.508)	**t127 (12)**	-	-	t321 (1); t8173 (1)	ST1 (14)	CC1 (14)
CC005	**12** (2.6)	8	0.359 (0.201-0.517)	t005 (4)	t006 (2)	-	t310 (1); t3749 (1); t5951 (1); t790 (1); t025 (1); t032 (1)	ST22 (7); ST737 (2); ST2286 (1); ST1806 (1); ST25 (1)	CC22 (10); Singleton (1)
CC065	**8** (1.7)	5	0.249 (0.102-0.395)	t350 (3)	t330 (2)	-	t065 (1); t2429 (1); t004 (1)	ST45 (8)	CC45 (8)
CC216	**6** (1.3)	3	0.190 (0.056-0.324)	t216 (2); t316 (2); t5391 (2)	-	-	-	ST59 (5); ST2282 (1)	CC59 (6)
CC280	**5** (1.1)	3	0.159 (0.034-0.285)	t280 (3)	-	-	t396 (1); t1102 (1)	ST25 (4); ST1017 (1)	CC25 (4); CC45 (1)
Others (CC no founders; Singletons; Excluded)	**62** (13.3)	40	0.968 (0.948-0.988)	t884 (7)	t100 (6); t164 (6)	t314 (3)	t1076 (2); t1546 (2); t3348 (2); t126 (2); t1442, t1065, t1071, t10942, t111, t118, t1509, t1537, t1538, t1552, t1897, t213, t2353, t287, t355, t458, t4640, t535, t587, t616, t693, t771, t819, t8467, t9527, t9560, t9602, t9787, t9864, t9865, t9866; t7820 (one each)	ST9 (10); ST34 (9); ST20 (6); ST121 (4); ST72 (4); ST15 (3); ST12 (2); ST45 (2); ST5 (2); ST1, ST152, ST1802, ST1808, ST22, ST2284, ST2290, ST2294, ST2358, ST2359, ST25, ST27, ST291, ST30, ST398, ST573, ST580, ST707, ST718, ST944 (one each)	CC9 (13): CC30 (10); CC20 (6); CC121 (5); CC72 (4); C15 (3); CC12 (2); CC1 (2); CC5 (2); CC22 (2); CC25 (2); ST45 (2); CC152 (1); CC398 (2); CC8 (1); CC707 (1); CC182 (1); CC no founder (1); Singleton (2)

(1) *spa*-CC: spa clonal complex; (2) SID- Simpson's Index of Diversity; CI- Confidence Interval; (3) MLST-CC: MLST clonal complex.

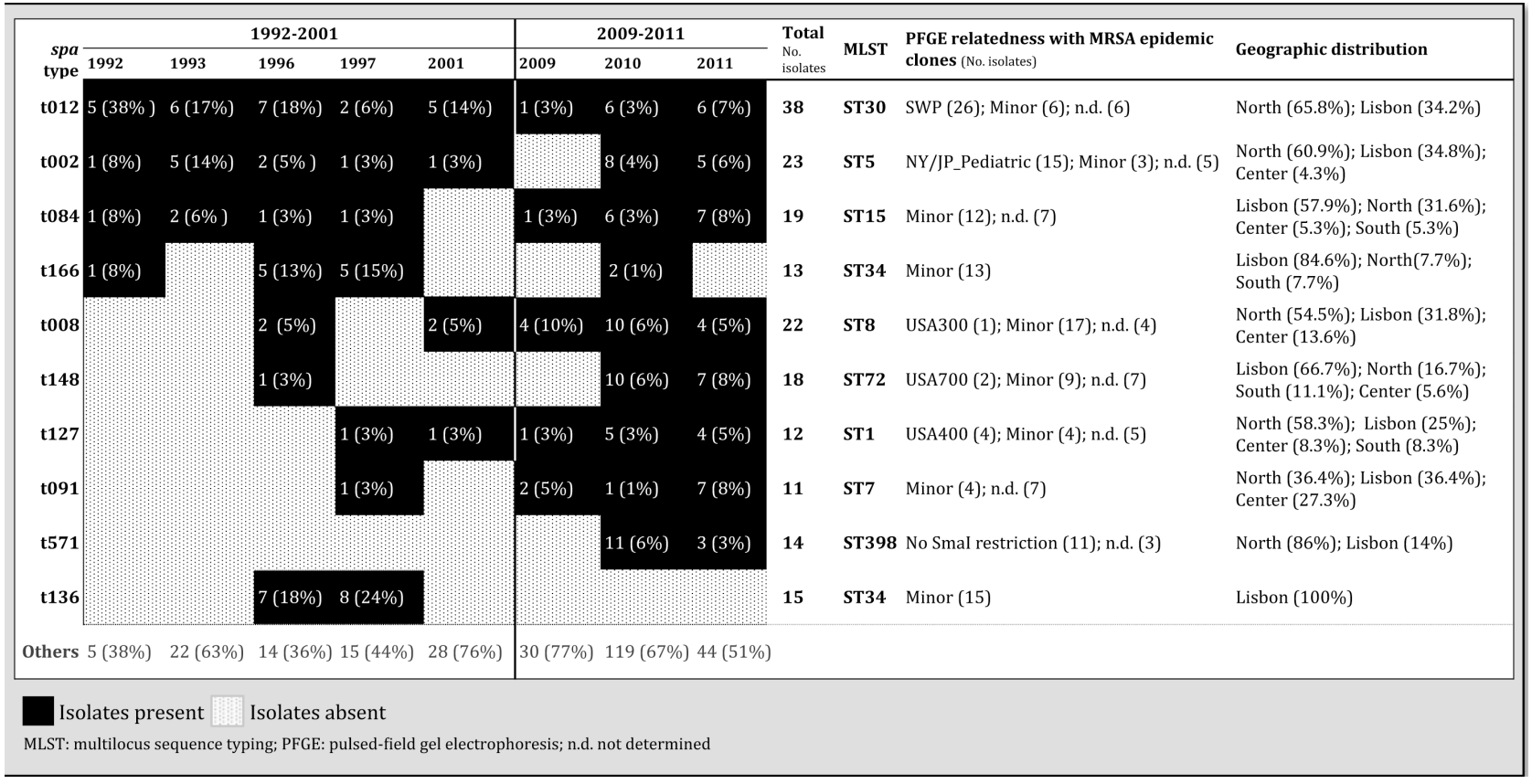
In terms of diversity, the *spa*-CCs showed heterogeneity, comprising from 2 to 17 different *spa* types (See Table 1). The most diverse clonal complexes were spa-CC015 [SID=0.881(0.832-0.930)], spa-CC084 [SID=0.819(0.760-0.877)], spa-CC002 [SID=0.805(0.728-0.881)], spa-CC008 [SID=0.774(0.700-0.848)] and spa-CC166 [SID=0.774(0.718-0.830)].

Among all isolates, the most predominant clonal types were ST30-t012 (8.2%, n=38), ST5-t002 (4.9%, n=23), ST8-t008 (4.7%, n=22), ST15-t084 (4.1%, n=19), ST72-t148 (3.9%, n=18), ST34-ST136 (3.2%, n=15), ST398-t571 (3%, n=14), ST34-t166 (2.8%, n=13), ST1-t127 (2.6%, n=12) and ST7-t091 (2.4%, n=11) (See Table 1 and Figure 2).

MSSA distribution in community and hospital

The six most common STs in the community and in the hospital were the same, including ST34, ST30, ST5, ST8, ST15 and ST45. However they showed different proportions in the two settings. The CO isolates were distributed as follows: ST34 (11.7%, n=39), ST30 (9%, n=30), ST5 (9%, n=30), ST8 (7.2%, n=24), ST15 (6.3%, n=21), ST45 (6%, n=20), ST121 (n=5.4%, n=18), ST72 (5.1%, n=17), ST398 (4.8%, n=16), ST25 (3.6%, n=12), ST1 (3%, n=10), ST22 (2.4%, n=8), ST9 (2.4%, n=8), ST7 (2.1%, n=7) and the remaining 21.9% isolates included 52 different STs. On the other hand, the HA isolates were represented by the ST30 (22%, n=27), ST5 (13%, n=16), ST15 (9.8%, n=12), ST45 (8.1%, n=10), ST8 (7.3%, n=9), ST34 (5.7%, n=7), ST121 (5.7%, n=7), ST72 (4.9%, n=6), ST1 (4.1%, n=5), ST7 (3.3%, n=4), ST25 (3.3%, n=4), ST97 (2.4%, n=3), ST188 (2.4%, n=3), ST9 (1.6%, n=2) with the remaining 6.5% isolates represented by seven STs. Although the proportion of the dominant STs shared by isolates from the hospital and community varied, only ST30 showed to be particularly associated to a specific environment, being significantly more represented in the hospital ($p<0.001$) than in the community. Moreover, the three STs ST398, ST59 and ST22 typically associated with epidemic CA and HA-MRSA clones, respectively, were only observed has belonging to the community ($p=0.01$, $p=0.17$ and $p=0.08$ respectively).

Considering the *spa*-CCs clustering analysis, we observed that most *spa*-CC were equally represented by hospital and community isolates, however, *spa*-CC012 included more HA isolates ($p<0.001$) and *spa*-CC166 and *spa*-CC005 included more ($p=0.01$) or exclusively CO isolates ($p=0.03$), respectively.



spa type	1992-2001					2009-2011			Total No. isolates	MLST	PFGE relatedness with MRSA epidemic clones (No. isolates)	Geographic distribution
	1992	1993	1996	1997	2001	2009	2010	2011				
t012	5 (38%)	6 (17%)	7 (18%)	2 (6%)	5 (14%)	1 (3%)	6 (3%)	6 (7%)	38	ST30	SWP (26); Minor (6); n.d. (6)	North (65.8%); Lisbon (34.2%)
t002	1 (8%)	5 (14%)	2 (5%)	1 (3%)	1 (3%)		8 (4%)	5 (6%)	23	ST5	NY/JP_Pediatric (15); Minor (3); n.d. (5)	North (60.9%); Lisbon (34.8%); Center (4.3%)
t084	1 (8%)	2 (6%)	1 (3%)	1 (3%)		1 (3%)	6 (3%)	7 (8%)	19	ST15	Minor (12); n.d. (7)	Lisbon (57.9%); North (31.6%); Center (5.3%); South (5.3%)
t166	1 (8%)		5 (13%)	5 (15%)			2 (1%)		13	ST34	Minor (13)	Lisbon (84.6%); North(7.7%); South (7.7%)
t008			2 (5%)		2 (5%)	4 (10%)	10 (6%)	4 (5%)	22	ST8	USA300 (1); Minor (17); n.d. (4)	North (54.5%); Lisbon (31.8%); Center (13.6%)
t148			1 (3%)				10 (6%)	7 (8%)	18	ST72	USA700 (2); Minor (9); n.d. (7)	Lisbon (66.7%); North (16.7%); South (11.1%); Center (5.6%)
t127				1 (3%)	1 (3%)	1 (3%)	5 (3%)	4 (5%)	12	ST1	USA400 (4); Minor (4); n.d. (5)	North (58.3%); Lisbon (25%); Center (8.3%); South (8.3%)
t091				1 (3%)		2 (5%)	1 (1%)	7 (8%)	11	ST7	Minor (4); n.d. (7)	North (36.4%); Lisbon (36.4%); Center (27.3%)
t571							11 (6%)	3 (3%)	14	ST398	No SmaI restriction (11); n.d. (3)	North (86%); Lisbon (14%)
t136			7 (18%)	8 (24%)					15	ST34	Minor (15)	Lisbon (100%)
Others	5 (38%)	22 (63%)	14 (36%)	15 (44%)	28 (76%)	30 (77%)	119 (67%)	44 (51%)				

MLST: multilocus sequence typing; PFGE: pulsed-field gel electrophoresis; n.d. not determined

Figure 2. Temporal and geographical distribution of the ten most predominant *spa* types.

Overall, the distribution of predominant clonal types varied in the CA and HA isolates. Whereas in the community the most frequent clonal type was ST8-t008 (3%, n=10), followed by ST398-t571 (3%, n=10), ST15-t084 (2%, n=7), ST30-t012 (2%, n=7) and ST7-t091 (2%, n=7); in the hospital the most frequent clonal types were ST30-t012 (15%, n=19), ST5-t002 (6%, n=8), ST15-t084 (5%, n=6), ST72-t148 (4%, n=5) and ST8-t008 (4%, n=5). The clonal type ST398-t571 was significantly associated to the community (p=0.05), whereas ST5-t002 was associated to the hospital (p=0.01).

MSSA predominant clonal types: dynamics overtime and geography

Regarding the dynamics of the MSSA population we observed the occurrence of three different patterns overtime: clonal types that were endemic in Portugal during the 19 years of the study; clonal types that were detected intermittently over the 19 years; and clonal types that appeared more recently only and were detected intermittently after their emergence (Figure 2).

The only clonal type that was endemic during the 19 years was clonal type ST30-t012. Other clonal were found intermittently; for example the clonal types ST5-t002 and ST15-t084 were sampled in all except one year (2009 and 2001 respectively) and ST34-t166 was absent in four sampling periods (1993, 2001, 2009, 2011); on the other hand, clonal type ST34-t136 was sampled only in two consecutives years (1996/97).

From 1996 on, five new MSSA clonal types were detected, the ST8-t008, ST72-t148, ST1-t127, ST398-t571 and ST7-t091 related to the CA-MRSA epidemic clones USA300, USA700, USA400, ST398-V and a minor CA clone, respectively. Altogether, these MSSA clonal types constitute nowadays 23% of the entire MSSA population, and, individually, comprise currently the most predominant clonal types, together with ST15-t084, ST5-t002 and ST30-t012.

Overall, a higher genetic diversity was observed in 2009/2011 [SID= 0.982 (0.977-0.987)] comparing to the early period 1992-2001 [SID= 0.956 (0.939-0.972)].

In what respects to the geographic distribution of MSSA in Portugal, we observed that, with the exception of ST34-t136, found exclusively in Lisbon, the remaining predominant *spa* types were disseminated all over the country (See Figure 2). Noteworthy, from the broadly dispersed clonal types,

the ST34-t166 (p=0.003) and ST72-t148 (p=0.016) were more predominant in Lisbon and ST30-t012 (p=0.0325), ST398-t571 (p=0.005) in the Northern region.

Relatedness of MSSA with locally established MRSA

The analysis of the isolates with predominant *spa* types for which PFGE was performed (n=142) (see Figure 1) revealed that 33.8% of these isolates (n=48) had more than 80% of similarity with prototype strains belonging to CA and HA-MRSA reference clones, namely: 68.4% (26 out of 38) of isolates with *spa* type t012 were similar to the Southwest Pacific (SWP) clone; 65.2% (15 out of 23) of isolates with t002 were similar to NY/JP and Pediatric clones; 33.3% (4 out of 12) of isolates t127 were similar to USA400 clone; 11.1% (2 out of 18) of isolates with t148 were similar to USA700 clone and 4.5% (1 out of 22) of isolates with t008 were similar to USA300 clone (See Figure 2).

DISCUSSION

In the present study, we attempted to contribute to the understanding of MSSA epidemiology, by analyzing the evolution of MSSA population along 19 years in Portugal. Moreover, we compared the relatedness of the established MSSA population with hospital and community-associated MRSA in the country.

We found that, despite the wide genetic diversity observed among the MSSA, a great proportion of the population belonged to ten major clonal types (40%), namely ST30-t012 (8.2%), ST5-t002 (4.9%), ST8-t008 (4.7%), ST15-t084 (4.1%), ST72-t148 (3.9%), ST34-t166 (3.2%), ST398-t571 (3%), ST34-t136 (3%), ST1-t127 (2.6%) and ST7-t091 (2.4%). Except for ST72-t148 and ST398-t571, all these clonal types were previously identified among MSSA recovered in Portugal between 1992 and 2001, although in different proportions (1). Interestingly, with exception of ST7-t091, ST398-t571 and ST34-t136, these same clonal types, were also frequently found in other European countries such as Spain (4) Belgium (21), The Netherlands (27), Germany (25) and in the USA (24) when analyzing MSSA isolates from different time periods. These results may suggest the existence of specific MSSA clones with increased endemicity and epidemicity.

On the other hand, in other countries like Taiwan, in Asia, and Cameroon, Madagascar, Morocco, Niger and Senegal in Africa, the MSSA population from carriage and infection in the community was different from that found in Portugal. In Taiwan ST508 (SLVST45)-t015, ST25-t340, ST121-t645, ST59-t437 and ST188-t189 were predominant (7) whereas in five major African cities in Cameroon, Madagascar, Morocco, Niger and Senegal, 58% of MSSA isolates from infection belonged to clones ST121-t314, ST15-t084, ST1851(SLV of ST1)-t127 and ST152-t355 (6). The reasons lying behind the variation in MSSA population structure between the different countries are poorly understood.

When analyzing the MSSA population structure over time we found that one of the most predominant clonal types, ST30-t012 was detected over the entire 19 years study period, whereas others were intermittently detected. The clone (ST30-t012) was detected for the first time more than 60 years ago, in the form of the penicillin-resistant phage-type 80/81 clone, and was shown to be highly epidemic and virulent. Finally more recently, has emerged also as contemporary CA-MRSA epidemic Southwest clone (ST30-IV, t012) (11).

In addition to ST30-t012, clonal types ST5-t002, ST8-t008, ST1-t127 were also already present in 1957, among the genetic backgrounds of the historically early MSSA (9, 19), collected in the UK and Denmark, suggesting they have been successful human clones for a long time.

We observed that clones like ST8-t008, ST30-t012, ST72-t148, ST1-t127 are highly related with the CA-MRSA epidemic clones recently described in Portugal, namely USA300 (ST8-IVa, t008), SWP (ST30-IVa, t012) USA700 (ST72-IVg, t127), USA400 (ST1-IVa, t148), respectively (30). With the exception of ST30-t012, these clonal types were all detected only after 1996 in our study. This time period coincides exactly with the worldwide emergence of CA-MRSA epidemic, which suggest that the MRSA belonging to these clonal types may have arisen from the local SCC*mec* IV acquisition giving rise to the country emergence of the CA-MRSA clones. Alternatively, they might result from the loss of SCC*mec* IV from CA-MRSA imported from abroad. The relatedness of contemporary MRSA and MSSA strains belonging to these clonal types was further confirmed by the high similarity of their SmaI PFGE macrorestriction profiles. This relatedness of contemporary MSSA and MRSA strains was previously observed in other countries (25). In particular, in the USA, where CA-MRSA are epidemic in both hospitals and community, the dominant MSSA clonal types found (ST5-t002 and ST8-t008) were analogous to the predominant MRSA clones (NY/JP and USA300 clones) (24). These results suggest that emergence of CA-MRSA might have contributed to a change both in MRSA and MSSA epidemiology.

Despite of the characteristic genetic diversity found among the MSSA population, dominant lineages seems to be established for more than 19 years and widely distributed in Portugal. The emergence of CA-MRSA might have had impact on the recent MSSA epidemiology in this country. More detailed studies are needed, namely through the application of WGS, to understand the level of relatedness of contemporary MSSA and CA-MRSA.

ACKNOWLEDGMENTS

We thank the Portuguese health care institutions that participated in the SRL (staphylococcal reference laboratory) project in collaboration with Professor Hermínia de Lencastre (Laboratory of Molecular Genetics, Member of the European Staphylococcal Reference Laboratory Working Group) that led to the isolation 87 MSSA isolates included in this study.

The experimental work was done by A. Tavares, with the exception of the molecular characterization of 87 out of 465 MSSA, which were performed by M. Aires de Sousa and N. A. Faria.

FUNDING

This study was funded by Project Ref. P-99911 from Fundação Calouste Gulbenkian, Lisbon, Portugal and grant Ref. Pest-OE/EQB/LAO004/2011, from Fundação para Ciência e Tecnologia (FCT), Portugal. A. Tavares was supported by grant SFRH/BD/44220/2008 from Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

REFERENCES

1. **Aires de Sousa, M., T. Conceicao, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community. J Clin Microbiol **43:**5150-7.
2. **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.
3. **Aires-de-Sousa, M., B. Correia, and H. de Lencastre.** 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:**2912-7.
4. **Argudin, M. A., V. Argumosa, M. C. Mendoza, B. Guerra, and M. R. Rodicio.** 2013. Population structure and exotoxin gene content of methicillin-susceptible *Staphylococcus aureus* from Spanish healthy carriers. Microb Pathog **54:**26-33.
5. **Bens, C. C., A. Voss, and C. H. Klaassen.** 2006. Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis. J Clin Microbiol **44:**1875-6.
6. **Breurec, S., C. Fall, R. Pouillot, P. Boisier, S. Brisse, F. Diene-Sarr, S. Djibo, J. Etienne, M. C. Fonkoua, J. D. Perrier-Gros-Claude, C. E. Ramarokoto, F. Randrianirina, J. M. Thiberge, S. B. Zriouil, B. Garin, and F. Laurent.** 2011. Epidemiology of methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of Panton-Valentine leukocidin genes. Clin Microbiol Infect **17:**633-9.
7. **Chen, F. J., L. K. Siu, J. C. Lin, C. H. Wang, and P. L. Lu.** 2012. Molecular typing and characterization of nasal carriage and community-onset infection methicillin-susceptible *Staphylococcus aureus* isolates in two Taiwan medical centers. BMC Infect Dis **12:**343.
8. **Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J. H. Song, P. T. Tassios, and P. Villari.** 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.
9. **Crisostomo, 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.
10. **David, M. Z., S. Boyle-Vavra, D. L. Zychowski, and R. S. Daum.** 2011. Methicillin-susceptible *Staphylococcus aureus* as a predominantly healthcare-associated pathogen: a possible reversal of roles? PLoS One **6:**e18217.
11. **DeLeo, F. R., A. D. Kennedy, L. Chen, J. Bubeck Wardenburg, S. D. Kobayashi, B. Mathema, K. R. Braughton, A. R. Whitney, A. E. Villaruz, C. A. Martens, S. F. Porcella, M. J. McGavin, M. Otto, J. M. Musser, and B. N. Kreiswirth.** 2011. Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus*. Proc Natl Acad Sci U S A **108:**18091-6.
12. **ECDC.** 2011. European Centre for Disease Prevention and Control. Antimicrobial Resistance Surveillance in Europe 2011. Available: http://www.ecdc.europa.eu/en/publications/Publications/Forms/ECDC_DispForm.aspx?ID=998.
13. **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.
14. **Espadinha, D., N. A. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, and H. de Lencastre.** 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.

15. **Faria, N. A., J. A. Carrico, 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.
16. **Faria, N. A., M. Miragaia, H. de Lencastre, and C. Multi Laboratory Project.** 2013. Massive dissemination of methicillin resistant *Staphylococcus aureus* in bloodstream infections in a high MRSA prevalence country: establishment and diversification of EMRSA-15. Microb Drug Resist **19:**483-90.
17. **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.
18. **Goering, R. V., R. M. Shawar, N. E. Scangarella, F. P. O'Hara, H. Amrine-Madsen, J. M. West, M. Dalessandro, J. A. Becker, S. L. Walsh, L. A. Miller, S. F. van Horn, E. S. Thomas, and M. E. Twynholm.** 2008. Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials. J Clin Microbiol **46:**2842-7.
19. **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.
20. **Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med **7:**e1000215.
21. **Hallin, M., O. Denis, A. Deplano, R. De Mendonca, R. De Ryck, S. Rottiers, and M. J. Struelens.** 2007. Genetic relatedness between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*: results of a national survey. J Antimicrob Chemother **59:**465-72.
22. **Lina, G., Y. Piemont, 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.
23. **Mellmann, A., T. Weniger, C. Berssenbrugge, J. Rothganger, 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.
24. **Miko, B. A., C. A. Hafer, C. J. Lee, S. B. Sullivan, M. A. Hackel, B. M. Johnson, S. Whittier, P. Della-Latta, A. C. Uhlemann, and F. D. Lowy.** 2013. Molecular characterization of methicillin-susceptible *Staphylococcus aureus* clinical isolates in the United States, 2004 to 2010. J Clin Microbiol **51:**874-9.
25. **Nulens, E., E. E. Stobberingh, H. van Dessel, S. Sebastian, F. H. van Tiel, P. S. Beisser, and R. H. Deurenberg.** 2008. Molecular characterization of *Staphylococcus aureus* bloodstream isolates collected in a Dutch University Hospital between 1999 and 2006. J Clin Microbiol **46:**2438-41.
26. **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.
27. **Rijnders, M. I., R. H. Deurenberg, M. L. Boumans, J. A. Hoogkamp-Korstanje, P. S. Beisser, and E. E. Stobberingh.** 2009. Population structure of *Staphylococcus aureus* strains isolated from intensive care unit patients in the netherlands over an 11-year period (1996 to 2006). J Clin Microbiol **47:**4090-5.
28. **Sa-Leao, R., I. S. Sanches, I. Couto, C. R. Alves, and H. de Lencastre.** 2001. Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist **7:**237-45.
29. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, 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.
30. **Tavares, A., M. Miragaia, J. Rolo, C. Coelho, and H. de Lencastre.** 2013. High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* in the community in Portugal: evidence for the blurring of community-hospital boundaries. Eur J Clin Microbiol Infect Dis.

31. **Weidenmaier, C., C. Goerke, and C. Wolz.** 2012. *Staphylococcus aureus* determinants for nasal colonization. Trends Microbiol **20:**243-50.
32. **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.

CHAPTER IV

Insights into alpha-hemolysin (Hla) evolution and expression among *Staphylococcus aureus* clones with hospital and community origin

THIS CHAPTER WAS PUBLISHED IN:

Tavares, A., J. B. Nielsen, K. Boye, S. Rohde, A. C. Paulo, H. Westh, K. Schonning, H. de Lencastre, and M. Miragaia. 2014. Insights into alpha-hemolysin (Hla) evolution and expression among *Staphylococcus aureus* clones with hospital and community origin. PLoS One **9:**e98634

ABSTRACT

Background

Alpha-hemolysin (Hla) is a major virulence factor in the pathogenesis of *Staphylococcus aureus* infection, being active against a wide range of host cells. Although *hla* is ubiquitous in *S. aureus*, its genetic diversity and variation in expression in different genetic backgrounds is not known. We evaluated nucleotide sequence variation and gene expression profiles of *hla* among representatives of hospital (HA) and community-associated (CA) *S. aureus* clones.

Methods

51 methicillin-resistant *S. aureus* and 22 methicillin-susceptible *S. aureus* were characterized by PFGE, *spa* typing, MLST and SCC*mec* typing. The internal regions of *hla* and the *hla* promoter were sequenced and gene expression was assessed by RT-PCR.

Results

Alpha-hemolysin encoding- and promoter sequences were diverse, with 12 and 23 different alleles, respectively. Based on phylogenetic analysis, we suggest that *hla* may have evolved together with the *S. aureus* genetic background, except for ST22, ST121, ST59 and ST93. Conversely, the promoter region showed lack of co-evolution with the genetic backgrounds. Four non-synonymous amino acid changes were identified close to important regions of *hla* activity. Amino acid changes in the RNAIII binding site were not associated to *hla* expression. Although expression rates of *hla* were in general strain-specific, we observed that CA clones showed significantly higher *hla* expression ($p=0.003$) when compared with HA clones.

Conclusion

We propose that the *hla* gene has evolved together with the genetic background. Overall, CA genetic backgrounds showed higher levels of *hla* expression than HA, and a high strain-to-strain variation of gene expression was detected in closely related strains.

INTRODUCTION

Staphylococcus aureus is a human opportunistic pathogen responsible for a wide range of infections that can vary in its clinical presentation and severity. Methicillin-resistant *S. aureus* (MRSA) emerged in 1960 in the United Kingdom and has been a major problem in hospitals (HA-MRSA) worldwide during the last 40 years; however since the late 1990s, MRSA has been emerging as a leading cause of severe infection also in the community, in individuals without recent health-care contact (CA-MRSA) (12, 16)

CA-MRSA present distinct genetic backgrounds from their hospital counterparts, are more susceptible to antibiotics other than beta-lactams, carry the smallest staphylococcal cassette chromosome *mec* types (SCC*mec* IV or V), and have higher virulence capacity (12, 16, 35). The underlying reasons behind the enhanced virulence of CA-MRSA appear to be multiple including a different capacity to overcome host cell response (20), different distribution of mobile genetic elements carrying virulence determinants (3), allelic variation in virulence determinants located in the core genome and in mobile genetic elements (17), and different levels of expression and protein production of virulence determinants (alpha-hemolysin, collagen adhesin, staphylokinase, coagulase, lipase, enterotoxins C3 and Q, V8 protease and cysteine protease) (8, 22, 26).

The alpha-hemolysin or α-toxin (Hla), is one of the major virulence determinants implicated in the pathogenesis of *S. aureus*, associated to severe skin and soft tissue infections (SSTI), necrotizing pneumonia and even sepsis (5). Hla is the most prominent *S. aureus* cytotoxin that can act against a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes and macrophages (5, 42, 46). The gene encoding Hla is located in the core genome and is expressed as a water-soluble monomer (33.2 kD) that assembles to form a membrane-bound heptameric β-barrel pore (232.4 kD) on susceptible cells leading to cell death and lysis (42). The overall structure is mushroom-like, divided into three domains: 1) Cap domain: largely hydrophobic, defining the entry of the pore; 2) Rim domain: underside of the Cap, in close proximity to membrane bilayer; 3) Stem domain: part of the transmembrane channel, forming the membrane-perforating β-barrel pore (Figure 1) (5, 42).

Hla expression is mainly controlled by the global toxin accessory gene regulator (*agr*), via the regulatory effector molecule RNAIII (32). While *agr* provides the first and most important mechanism of up-regulation of *hla*, expression can also be modulated by other regulators, such as SaeR, SarZ, ArlS (4, 24, 25) (up-regulators) and Rot, SarT (41)(down-regulators).

Although polymorphisms in the *hla* promoter region have been described (23), the range of genetic diversity and evolution of this toxin has never been assessed in a large representative *S. aureus* collection. Furthermore, although differences in *hla* expression have been described between community- and hospital-associated MRSA, these studies have been performed with a limited number of CA-MRSA epidemic clones (22), or almost exclusively with representatives of the USA300 clone (6, 7, 19). To better understand the evolutionary history of *hla* and its importance as a virulence factor for CA-MRSA, in this study we compared the *hla* nucleotide sequence and expression among the major epidemic and minor CA and HA clones, including both MRSA and MSSA strains.

MATERIALS AND METHODS

Ethics Statement

Isolates were obtained from routine diagnostic and were analyzed anonymously and only the isolates, not humans, were studied. All data was collected according to the European Parliament and Council decision for the epidemiological surveillance and control of communicable disease in the European community. Ethical approval and informed consent were for that reason not required.

Bacterial collection

A total of 73 *S. aureus*, including 51 MRSA and 22 MSSA were analyzed in this study. Strains were collected in 13 different countries (Belgium, Bulgaria, Czech Republic, Denmark, Greece, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom, USA and Brazil), between 1961 and 2009 from both community (n=46) and hospital (n=27). The strains comprised a total of 52 *spa* types and 23 sequence types (STs) (see Table S1).

Strains were defined as belonging to CA or HA clones if they contained the same or related genetic backgrounds as the reference CA-MRSA and HA-MRSA epidemic control strains, based on ST, *spa* type and SCC*mec* (in case of MRSA).

Media and bacterial growth conditions

Before RT-PCR analysis, strains were grown overnight at 37°C on tryptic soy agar plates (TSA). Bacterial growth experiments were performed by growing bacteria in tryptic Soy Broth (TSB) at 37°C with shake and measuring OD (600 nm) each hour in the follow up automatic incubator Bioscreen C (Oy Growth Curves AB, Helsinki, Finland). Plates of 100-well honeycomb (Oy Growth Curves AB, Helsinki, Finland) were filled with 300µl/well of overnight culture diluted to OD_{600}=0.05 in TSB growth medium. Three individual growth experiments (SetC, SetD and SetE) were performed for each strain and named accordingly e.g. HLZ6C, HLZ6D and HLZ6E (see Figure S2.I to III).

Nucleotide sequence of *hla* and promoter region

Chromosomal DNA was extracted from overnight cultures, using the boiling method (100°C for 10 min followed by centrifuged at 13.000g for 5 minutes). Two sets of primers were designed to span the most polymorphic regions within the *hla* gene and *hla* promoter (considered as the region located -600 bp from *hla* starting codon), after alignment of sequences available on NCBI for *S. aureus*. One set of

primers (Forward: hla-F_CGAAAGGTACCATTGCTGGT; Reverse: hla-R_CCAATCGATTTTATATCTTTC) amplified an internal fragment of the *hla* gene (nt 1170419-1170982, CP000730.1) and the other set (Forward: hlaPro-F_CACTATATTAAAAATACATAC; Reverse: hlaPro-R_GTTGTTACTGAGCTGAC) amplified an internal fragment of the *hla* promoter region (nt 1171289-1171773, CP000730.1) (Figure S3). PCR products were sequenced (Macrogen Europe, Amsterdam, The Netherlands) and sequences were analyzed using SeqMan (DNAstar, Lasergene v9, Madison, WI, USA). To each unique *hla* promoter (P) and gene sequence (*hla*) - allotype - a single Arabic number was attributed (e.g. P1, P2; *hla*1, *hla*2). Gene and promoter sequences were deposited in GenBank (accession numbers KM019547-KM019606; KM019607-KM019674).

Phylogenetic analysis

Phylogenetic relatedness was analyzed using the MEGA5 v5.05 software (http://www.megasoftware.net/) for gene, promoter region and concatenated sequences obtained from 1) gene with promoter region and 2) seven MLST alleles from the 23 representative STs within the collection. Phylogenetic trees were constructed using the Neighbor-Joining clustering method, and 1000 bootstrap replicates, which assigns confidence values for the groupings in the tree.

Moreover, nucleotide diversity (ND) between the two clusters was calculated based on the estimation of the average evolutionary divergence over sequence pairs within the two groups, where the number of base substitutions per site from averaging over all sequence pairs within each group are compared using the maximum composite likelihood model (43).

Detection of recombination

Alignments from the *hla* gene, *hla* promoter and internal fragments of each of the seven MLST gene were screened for the occurrence of putative recombination events using Recombination Detection Program version 4 (RDP4) (http://web.cbio.uct.ac.za/) with the default settings (with highest acceptable probability value of 0.05). Identification of recombinant sequences recombination breakpoints and major parent was determined using simultaneously nine recombination detection methods (RDP, BOOTSCAN, GENECONV, MAXCHI, CHIMAERA, SISCAN, PhylPro, LARD and 3SEQ. The “minor parent” is considered a sequence closely related to that from which sequences in the proposed recombinant region may have been derived (the presumed donor). The “major parent”

was considered as a sequence closely related to that from which the greater part of the recombinant's sequence may have been derived.

RT-PCR analysis

Culture growth was stopped at late exponential phase, when alpha-toxin is described to have maximal activity (47), corresponding to the time-points 1) 3 hours 30 min in one group (65 strains) and 2) 4 hours 30 min in another (8 strains). Total RNA was extracted from three biological replicates. Cells were mechanically disrupted with FastPrep-24 Instrument (MP Biomedicals, Solon, OH, USA) and RNA was protected using RNA Protect (Qiagen, Valencia, USA). RNA was extracted automatically using the QIAsymphony platforms (Qiagen, Valencia, USA) with QIAsymphony RNA kit (Qiagen, Valencia, USA).

The RT-PCR assay was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following primers and TaqMan probes: Hla RT_F: TAATGAATCCTGTCGCTAATGCC; HlaRT_R: CACCTGTTTTTACTGTAGTATTGCTTCC; Hla RT Probe: 6FAM-AAACCGGTACTACAGATAT-MGBNFQ. The RT-PCR reaction was performed using the EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, USA), in which RNA is reverse transcribed and amplified in a single reaction. The following PCR protocol was used: 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, followed by 42 cycles of 95°C for 20 sec and 62°C for 1 min. The 16S gene was used as internal or reference control. The primers used for 16S RNA amplification were those previously described (50).

RT-PCR data analysis

The relative *hla* gene expression was calculated based on the C_t (RT-PCR output) of the gene of interest (C_t hla) as compared to the C_t of the internal control (C_t 16S) as follows: Delta C_t = C_t hla- C_t 16S. The lower the Delta C_t the higher is the amount of *hla* mRNA and the more the gene is expressed. The reproducibility of the assay was evaluated by the calculation of the arithmetic mean of the relative expression of the three biological replicates (Mean Delta C_{t1-3}= Average (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}). The reproducibility of RT-PCR reaction was evaluated by the calculation of the standard deviation (STDEV) of Delta C_t obtained for each biological replica (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}). Values were considered valid when at least two C_t readings exist with STDEV<2.

Protein structure visualization (pyMOL)

The protein structure was modeled using PyMOL v.1.6 (http://www.pymol.org/) if a nucleotide mutation gave rise to a stop codon.

Statistical analysis

The statistical analysis was performed using the Graphpad Prism 6 (http://www.graphpad.com/scientific-software/prism/), with the two-tailed Student's t-test to determine whether the differences of mean expression rates (MSSA *versus* MSSA; HA backgrounds *versus* CA backgrounds) were statistically significant ($p \leq 0.05$).

Regression tree analysis was used to explore which variables could be related with the *hla* expression (14). Trees explain the variation of a single response variable (in this study the *hla* mRNA expression) by repeatedly splitting the data into more homogeneous groups, using combinations of explanatory variables (in our case, the ST, *spa* type, MRSA, MSSA and the type of SCC*mec*).

RESULTS

Analysis of polymorphisms in the *hla* gene and *hla* promoter

The sequence analysis of the internal region of *hla* and the *hla* promoter region among the 73 strains identified a total of 13 *hla* and 23 promoter region different sequences (allotypes) (Table 1). We obtained no amplification products for *hla* and *hla* promoter region in one and 13 strains, respectively, which probably result from misparing of the primers used.

From the 13 *hla* (*hla*1-13), we observed that only a single *hla*-allotype was found among representatives of a specific ST, except for ST22 (*hla*12; *hla*13) and ST30 (*hla*8; *hla*9) where two different alleles were identified. On the other hand, the most frequent alleles, *hla1* (33.3%, n=24) and *hla4* (20.8%, n=15), were identified in more than one ST.

Regarding the nucleotide changes identified in the *hla*, some correspond to non-synonymous mutations (E208, T239 and S243) and, in one particular case, to a stop codon (Table 1 and 2). The substitutions observed did not correspond to any difference in the charge or polarity of the amino acid (aa). However, changes in molecular weight were observed: i) changes from aa D208 to aa E208 (D208E) and from aa S239 to T239 (S239T) gave rise to a higher molecular weight aa; and ii) change from aa T243 to S243 (T243S) resulted in a lower molecular weight aa; of note all changes occurred in the Rim and Cap domain of the protein. In a particular case, the aa change gave rise to a stop codon located in the Cap domain, in strains of ST36 and one ST30. Protein structure modeling showed that a protein of about one third of its real size is produced, truncated at the Gln87 (Figure 1, A and B). The truncation is in the outside part of the domain, suggesting that this will affect the capacity of the Hla to form cell wall pores, and ultimately to induce hemolysis.

Table 1. Summary of molecular characterization, sequence variation and relative expression rates of *S. aureus* strains collection.

Nº	Isolate ID	SCC*mec*	*spa* type	MLST	Branch[1]	Promotor Allotype	Gene Allotype	Nonsynonymous Mutation	Hla Expression (Mean Delta C_t)[2]	Stddev Delta C_t[3]	Expression (High/Low)
1	HLZ6	II	t002	**ST5**	L	P4	hla1	D208E	8.69	2*	Low
2	BK2464	II	t002	**ST5**	L	nt	hla1	D208E	5.37	1	**High**
3	HBR73	II	t067	**ST5**	L	P5	hla1	D208E	8.75	1	Low
4	C013	VI	t002	**ST5**	L	P3	hla1	D208E	6.84	1	Low
5	HDES26	VI	t062	**ST5**	L	P3	hla1	D208E	8.01	1	Low
6	HDE288	VI	t311	**ST5**	L	P3	hla1	D208E	6.67	1	Low
7	HSA29	-	t002	**ST5**	L	P3	hla1	D208E	Not Valid	-	Not Valid
8	HDE461	IV	t022	**ST22**	H	P10	hla12	S239T; T243S	6.60	1	Low
9	HAR22	IV	t022	**ST22**	H	P11	hla13	S239T; T243S	6.43	1	Low
10	HSMB280	IV	t032	**ST22**	H	P10	hla12	S239T; T243S	4.71	1	**High**
11	LBM12	IV	t747	**ST1806**	H	nt	hla12	S239T; T243S	9.28	1	Low
12	HSMB184	-	t5951	**ST1806**	H	P10	hla12	S239T; T243S	6.74	1	Low
13	HPH2	II	t018	**ST36**	H	P7	hla8	D208E; S239T; stop codon	8.02	2*	Low
14	HAR24	II	t018	**ST36**	H	nt	hla8	D208E; S239T; stop codon	9.62	2*	Low
15	DEN4415	II	t021	**ST36**	H	P7	hla8	D208E; S239T; stop codon	8.95	2*	Low
16	C563	IV	t015	**ST45**	H	nt	hla10	S239T	7.02	1	Low
17	C036	V	t015	**ST45**	H	nt	hla10	S239T	6.24	0	Low
18	HAR38	IV	t004	**ST45**	H	P7	hla10	S239T	10.38	1	Low
19	HFX77	III	t037	**ST239**	L	P1	hla4	-	8.74	2*	Low
20	HUC343	IIIA	t037	**ST239**	L	P1	hla4	-	8.27	0	Low
21	HU25	IIIA	t138	**ST239**	L	P1	hla4	-	8.17	1	Low
22	BK1953	IA	t051	**ST247**	L	P1	hla4	-	7.71	1	Low
23	HPV107	IA	t051	**ST247**	L	P1	hla4	-	7.56	0	Low
24	HSJ419	IA	t725	**ST247**	L	P1	hla4	-	8.23	1	Low
27	E2125	I	t051	**ST247**	L	P1	hla4	-	7.29	0	Low
25	10395	I	t008	**ST250**	L	P2	hla4	-	8.15	1	Low
26	COL	I	t008	**ST250**	L	P1	hla4	-	8.01	1	Low
28	HFX74	IV	t008	**ST8**	L	P1	hla4	-	6.46	1	Low
29	USA300	IV	t008	**ST8**	L	P1	hla4	-	6.19	3*	Low
30	C438	IV	t024	**ST8**	L	P1	hla4	-	6.07	1	Low
31	C574B	IV	t1257	**ST612**	L	P1	hla4	-	Not Valid	-	Not Valid
32	LBM27	-	t024	**ST8**	L	P1	hla4	-	8.12	0	Low
33	LBM74	-	t008	**ST8**	L	P1	hla4	-	5.87	1	Low
34	C270	IV	t1381	**ST1**	L	P17	hla2	-	8.81	1	Low
35	USA400	IV	t127	**ST1**	L	P17	hla2	-	6.01	2*	Low
36	LBM36	-	t127	**ST1**	L	P18	hla2	-	11.09	1	Low
37	C577	IV	t216	**ST59**	L	P20	hla5	-	5.35	0	**High**
38	C583	IV	t437	**ST59**	L	P19	hla5	-	5.31	1	**High**
39	C434	V	t437	**ST59**	L	P19	hla5	-	9.14	1	Low
40	C018	IV	t1819	**ST93**	L	nt	hla7	-	5.16	1	**High**
41	C491	IV	t202	**ST93**	L	P21	hla7	-	5.45	0	**High**
42	LBM54	IV	t011	**ST398**	H	P12	hla11	-	4.46	2*	**High**
43	C482	IV	t011	**ST398**	H	P13	hla11	-	3.25	1	**High**
44	C496	VII	t108	**ST398**	H	nt	hla11	-	2.85	1	**High**
45	LBM40	-	t034	**ST398**	H	P12	hla11	-	5.37	1	**High**
46	C017	IV	t019	**ST30**	H	nt	hla9	D208E; S239T	4.53	0	**High**
47	C385	IV	t019	**ST30**	H	P7	hla9	D208E; S239T	7.25	1	Low
48	C479	IV	t019	**ST30**	H	nt	hla9	D208E; S239T	8.10	1	Low
71	HUC585	-	t342	**ST30**	H	P7	hla9	D208E; S239T	5.14	1	**High**
69	HFF204	-	t318	**ST30**	H	P9	hla9	D208E; S239T	6.23	1	Low
70	HFA30	-	t012	**ST30**	H	P8	hla8	D208E; S239T; stop codon	7.94	1	Low
49	HSJO7	IV	t148	**ST72**	L	P14	hla1	D208E	6.56	1	Low
50	USA700	IV	t148	**ST72**	L	P14	hla1	D208E	5.76	0	Low
51	COO3	IV	t791	**ST72**	L	P15	hla1	D208E	6.28	1	Low
52	SAMS1024	IV	t1346	**ST1810**	L	P14	hla1	D208E	4.78	1	**High**
53	HUC594	-	t148	**ST72**	L	P14	hla1	D208E	8.36	1	Low
54	HFA28	-	t126	**ST72**	L	P14	hla1	D208E	4.56	2*	**High**
55	C238	-	t3682	**ST72**	L	P14	hla1	D208E	4.64	1	**High**
56	C168	IV	t044	**ST80**	L	P16	hla1	D208E	8.20	0	Low
57	C485	IV	t044	**ST80**	L	P16	hla1	D208E	5.72	1	**High**
58	C014	IV	t131	**ST80**	L	P16	hla1	D208E	4.87	0	**High**
59	LBM25	-	t1509	**ST15**	L	P2	hla1	D208E	6.69	0	Low
60	C157	-	t084	**ST15**	L	P2	hla1	D208E	4.86	1	**High**
61	C230	-	t346	**ST15**	L	P2	hla1	D208E	9.03	2*	Low
62	HBA33	-	t258	**ST25**	L	P6	hla1	D208E	5.73	1	**High**
63	C095	-	t2909	**ST25**	L	P6	hla1	D208E	4.16	1	**High**
64	C141	-	t081	**ST25**	L	P6	hla1	D208E	4.50	2*	**High**
65	HBA34	IV	t308	**ST121**	L	nt	hla6	-	5.62	1	High
66	HUC574	-	t435	**ST121**	L	P1	hla6	-	5.19	1	**High**
67	HUC587	-	t159	**ST121**	L	P2	hla6	-	5.09	1	**High**
68	HUC578	-	t284	**ST121**	L	P1	hla6	-	7.10	2*	Low
72	LBM23	-	t100	**ST9**	L	P22	hla1	D208E	5.48	2*	**High**
73	HFX84	-	t267	**ST97**	L	P23	hla3	-	9.03	1	Low

(1) H: High polymorphism; L: Low polymorphism (2) Mean Delta $C_{t1\text{-}3}$= Average (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}), Delta C_t=C_t hla-C_t 16S; Not valid: only one C_t reading; (3) * low reproducibility between three C_T values (Stddv≤2). nt: non typable; Stddv: standard deviation

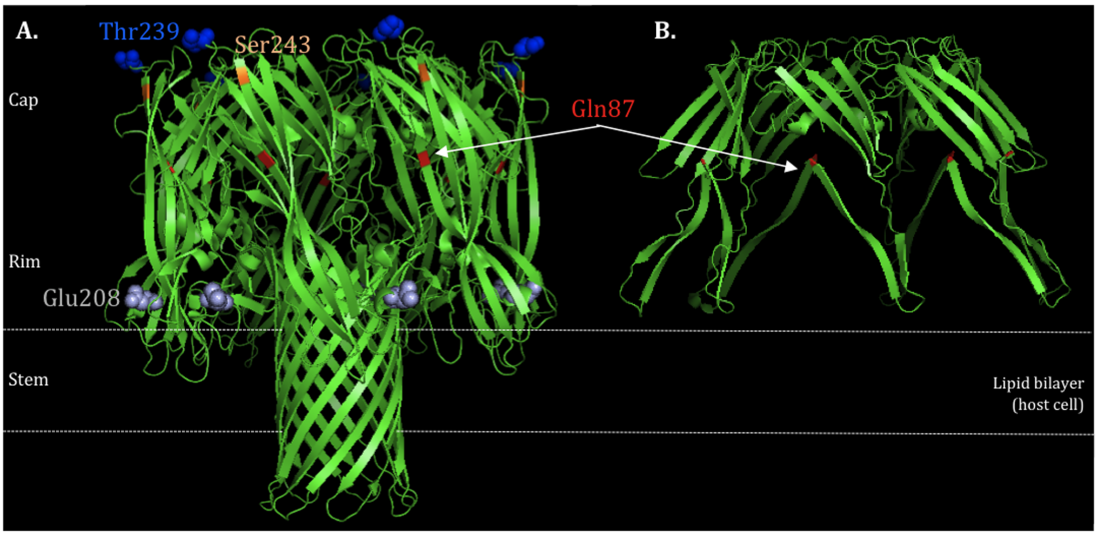


Figure 1. HLA protein structure. A) wildtype (highlighted the non-synonymous mutations Gln87, Glu208, Thr239 and Ser243) and B) truncated protein due to a stop codon at Gln87. Structure generated by the program PyMOL v.1.6.

A high number of sequence variations were identified in the *hla* promoter region, (n=23) (P1-23) (Table 1 and 2). Although we found that some STs were associated to a specific promoter allotype, and some promoters were identified in a single ST, we also identified cases where single STs were associated to different promoters (8 out of 23) and examples in which a single promoter allotype was associated to different STs (5 out of 23). This is the case of the most frequent promoter (P1) that was found in about one third of the strains analyzed (25.4%, n=16), including several different STs.

A particular highly polymorphic region corresponding to nt -22 to -24 from the start codon, was found in the majority (16 out of 23) of the promoter allotypes (exceptions P1, P6, P13, P14, P15, P18 and P23). These polymorphisms are located in the vicinity of RNAIII binding site (30); however, we could not find a direct correlation between a particular nucleotide sequence and a specific expression pattern (high or low expression). For example, the sequence T**T**T, observed in two strains belonging to ST398 that have a high level expression, was also observed in strains with low expression belonging to other genetic backgrounds (ST8, ST239, ST247, ST250, ST36, ST45 and ST22).

Alpha-hemolysin evolutionary history

In order to better understand the evolution of *hla* gene within the *S. aureus* population, we constructed phylogenetic trees from the *hla* and *hla* promoter sequences (Figure 2, A and Figure S2) and

compared it with the tree constructed from the concatenated sequences of the seven housekeeping genes used in MLST, including all the STs represented in the strain collection described here (Figure 2, B).

The phylogenetic tree constructed for the *hla* gene showed two distinct major clusters with different evolutionary clocks that differed in their nucleotide diversity (ND, see Materials and Methods): cluster (L) with lower diversity (ND=0.005), and cluster H with higher diversity (ND=0.019). Cluster L included more than 70% of strains (71.2%, n=52), and five sub-clusters; Cluster H contained about 29% of the strains (28.8%, n=21), and comprised four minor sub-clusters including *hla8*-*hla12* alleles, which were found in strains of ST30, ST36, ST45, ST398 and ST22.

As opposed to the phylogenetic tree constructed from *hla* gene, the one constructed from the promoter region did not show two distinct evolutionary branches (Figure S1). Moreover, dissimilar subgroup clustering was noticed in the tree constructed from the promoter gene sequence. For example, ST45, ST30 and ST36 backgrounds were clustered together in the promoter sequence-based tree whereas in the *hla* sequence-based tree ST45 was placed separately from ST30 and ST36 cluster (branch H). The same type of observations can be drawn for most of STs. Overall the promoter region showed to be more diverse than the *hla* gene sequence among the different backgrounds.

On the other hand, when we compared the phylogenetic tree constructed with the *hla* gene with that constructed from MLST concatenated genes, the same type of division into two distinct main clusters was observed (Figure 2). Moreover, the majority of STs were equally distributed between the two clusters in the two trees. The only exceptions were ST22, ST121, ST59 and ST93 that in the two trees have exchanged their positions from one cluster to the other (Figure 2, B-blue arrows).

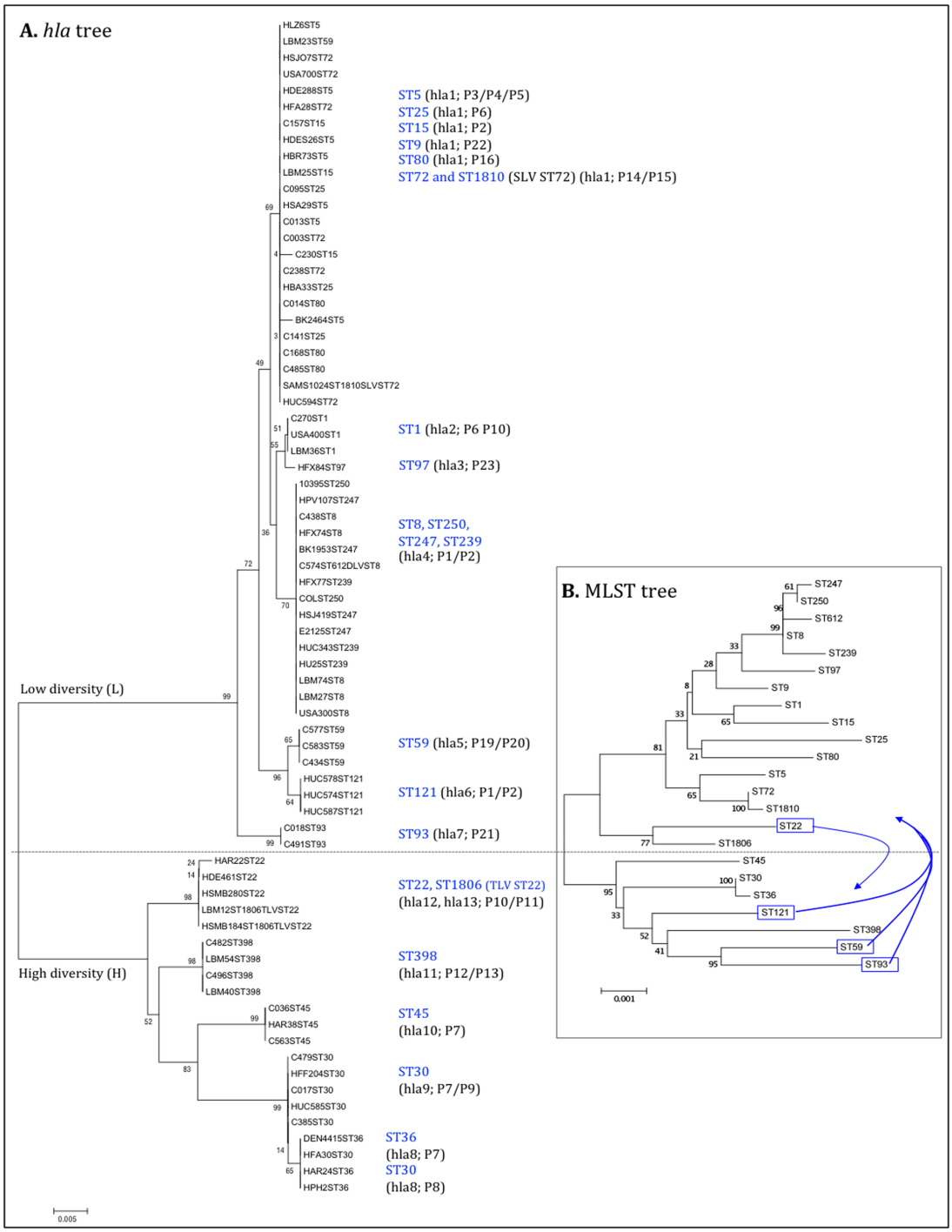


Figure 2. Phylogenetic trees of *hla* gene (A) and concatenated sequences of MLST alleles (B) from 23 STs representatives of the strains collection. The tree was constructed using MEGA 5 with Neighbour-joining method and bootstrap values provided as percents over 1000 replications. Branch length values are indicated and the percentage of replicate trees (bootstrap test) are shown next to the branches. The dashed line indicates the separation of the two evolutionary branches.

Detection of recombination in *hla* gene, h*la* promoter and MLST genes

To understand if recombination could explain the incongruence found between the trees constructed from *hla* and MLST concatenated genes, we screened the *hla* gene, *hla* promoter and each MLST gene for recombination events using the RDP4 software.

The SiScan and 3Seq methods detected one recombination event in the *hla* gene. This event corresponded to a fragment ending in positions 385-410 of the *hla* alignment, however the beginning breakpoint was not possible to determine. In the collection analyzed, this event was detected in five isolates belonging to ST22 or related STs (HSMB280, HDE461, HAR22 and LBM12 (TLV ST22) and HSMB184 (TLV ST22)) and four isolates of ST398 (LBM54, LBM40, C496, C482_ST398). The ST30 HFF204 strain was identified as the minor parent (97.8% identity with ST22 strains and 99.3% identity with ST398 strains) and ST121 strain HUC587 was identified as the major parent (with 100% identity to ST398 strains and 93.5-95.2% identity with ST22 strains) of the recombining fragment. A trace signal of recombination of this same event was also identified among ST45 isolates; however this signal was not statistically significant. Interestingly all the recombination events were detected in strains belonging to the high genetic diversity cluster in the tree constructed from *hla* gene. In the *hla* promoter region no recombination events were detected.

We have performed the same type of analysis using the internal sequences of each of the seven housekeeping used in MLST scheme, including the alleles present in all STs identified in this study, however no recombination events were detected in any of the genes.

Altogether the data gathered suggest that for the majority of strains *hla* gene evolved together with the genetic background. The different clustering of ST22 and ST121 strains, in the trees constructed from MLST concatenated genes and *hla* gene, may derive from recombination events occurring in the *hla* gene. Similarly these type of events might explain the genetic diversity observed in cluster H in the *hla* tree in strains belonging to ST22, ST398, ST45, ST30 and ST36 (H cluster of *hla* tree).

Expression of alpha-hemolysin

The expression of alpha-hemolysin in the 73 strains was assessed by RT-PCR, in three biological replicates. Fifteen of the 73 strains (20.5%) were excluded from the final analysis, either because a

single valid determination for Delta C_t (N=2) was obtained or because C_t obtained from the different biological replicates were not reproducible (N=13).

The analysis of the regression tree split the response variable into two distinct groups, according to the *spa* type of the strains. There was a group of strains with mean Delta $C_{t1-3} \leq 5.73$, that was classified as a high expression group and a second group with a mean Delta $C_{t1-3} > 5.73$ classified as a low expression group (Table 1, Table 2 and Figure 3). Overall the regression tree explained 60% of the variance in the data. This is mostly because there were strains expressing a low or high mean Delta C_t that were classified in the same *spa* type; those were the cases of *spa* types t002, t019, t044 and t437.

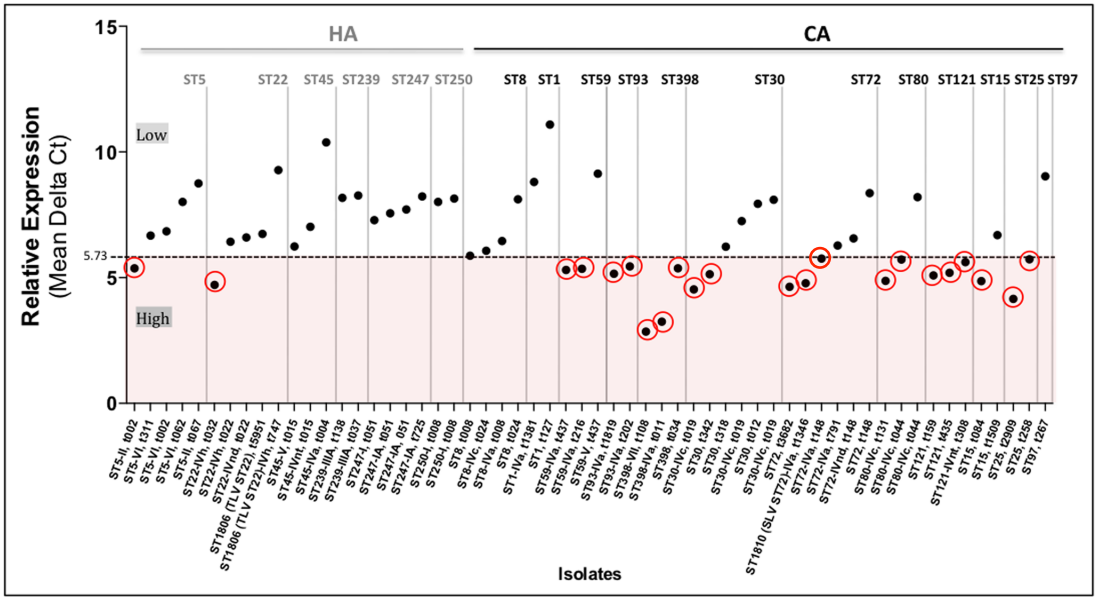


Figure 3. HA and CA strains relative expression distribution. Mean of expression rates from three biological replicates. Dashed line corresponding to the mean Ct value 5.73 results from the regression tree analysis which split strains in two distinct groups, at *spa* type level: a) high expression group - corresponding to strains with Mean Delta Ct≤ 5.73 and b) low expression group- corresponding to strains with Mean Delta Ct> 5.73). Highlighted in red are the high expressing strains.

Table 2. Strains data distribution based on promoter allotypes.

		Promotor allotype	Gene allotype	Non Synonymous Mutation	Isolates Molecular Characterization	Expression Category
CA backgrounds	ST398	P13	hla11	-	ST398-IV, t011	**High expression**
		P12			ST398, t034	**High expression**
		NT			ST398-VII, t108	**High expression**
		P12			ST398-IV, t011	**High expression***
	ST25	P6	hla1	D208E	ST25, t258	**High expression**
					ST25, t081	**High expression***
					ST25, t2909	**High expression**
	ST9	P22	hla1	D208E	ST9, t100	**High expression***
	ST93	P21	hla7	-	ST93-IV, t202	**High expression**
		NT			ST93-IV, t1819	**High expression**
	ST121	P2	hla6	-	ST121, t159	**High expression**
		P1			ST121, t435	**High expression**
		NT			ST121-IV, t308	**High expression**
		P1			ST121, t284	Low expression*
	ST72	P14	hla1	D208E	ST72-IV, t148	**High expression**
		P14			ST72, t3682	**High expression**
		P14			ST1810-IV, t1346	**High expression**
		P14			ST72, t126	**High expression***
		P15			ST72-IV, t791	Low expression
		P14			ST72-IV, t148	Low expression
		P14			ST72, t148	Low expression
	ST80	P16	hla1	D208E	ST80-IcV, t131	**High expression**
					ST80-IV, t044	**High expression**
					ST80-IV, t044	Low expression
	ST30	P7	hla9	D208E; S239T	ST30, t342	**High expression**
		NT			ST30-IV, t019	**High expression**
		P7			ST30-IV, t019	Low expression
		P9			ST30, t318	Low expression
		NT			ST30-IV, t019	Low expression
		P8	hla8	D208E; S239T; stop codon	ST30, t012	Low expression
	ST15	P2	hla1	D208E	ST15, t084	**High expression**
					ST15, t346	Low expression*
					ST15, t1509	Low expression
	ST59	P20	hla5	-	ST59-IV, t216	**High expression**
		P19			ST59-IV, t437	**High expression**
		P19			ST59-V, t437	Low expression
	ST1	P17	hla2	-	ST1-IV, t1381	Low expression
		P17			ST1-IV, t127	Low expression*
		P18			ST1, t127	Low expression
	ST8	P1	hla4	-	ST8-IV, t008	Low expression
					ST8-IV, t024	Low expression
					ST8-IV, t008	Low expression*
					ST8, t008	Low expression
					ST612-IV, t1257	Not valid**
					ST8, t024	Low expression
	ST97	P23	hla3	-	ST97, t267	Low expression
HA backgrounds	ST22	P10	hla13	S239T; T243S	ST22-IV, t032	**High expression**
		P10	hla12		ST22-IV, t022	Low expression
		P11			ST22-IV, t022	Low expression
		P10			ST1806, t5951	Low expression
		NT			ST1806-IV, t747	Low expression
	ST5	NT	hla1	D208E	ST5-II, t002	**High expression**
		P3			ST5-VI, t002	Low expression
		P3			ST5-VI, t062	Low expression
		P3			ST5-VI, t311	Low expression
		P4			ST5-II, t002	Low expression*
		P3			ST5, t002,	Not valid**
		P5			ST5-II, t067	Low expression
	ST36	P7	hla8	D208E; S239T; stop codon	ST36-II, t018	Low expression*
		P7			ST36-II, t021	Low expression*
		NT			ST36-II, t01	Low expression*
	ST45	NT	hla10	S239T	ST45-IV, t015	Low expression
		NT			ST45-V, t015	Low expression
		P7			ST45-IV, t004	Low expression
	ST239	P1	hla4	-	ST239-IIIA, t037	Low expression
					ST239-III, t037	Low expression*
				-	ST239-IIIA, t138	Low expression
	ST247	P1	hla4	-	ST247-I, t051	Low expression
					ST247-IA, 051	Low expression
					ST247-IA, t051	Low expression
					ST247-IA, t725	Low expression
	ST250	P1	hla4	-	ST250-I, t008	Low expression
		P2			ST250-I, t008	Low expression

(*)(**) relative expression values not valid (SDV≤2 or only one C_t reading)

Furthermore, we explored in each of the *spa* types what other explanatory variables (ST, MRSA, MSSA and type of SCC*mec*) could differentiate the inclusion of some strains in the low or high expression group, but we found no associations with the variables we measured in the study.

We observed that the *hla* expression level varied within strains of the same ST (Figure 3; Table 1 and 2). In fact, in some cases the same ST comprised strains with both high and low levels of expression (ST5, ST15, ST22, ST30, ST59, ST72 and ST80). Moreover, we found that the expression rates did not differ significantly (P=0.665) between MRSA and MSSA strains. However, we did find a correlation between the *hla* expression and the origin of the genetic backgrounds. Actually, strains of CA genetic backgrounds showed, in general, higher mean expression rates than strains of HA backgrounds (p=0.003) (Figure 4). Among the 22 strains (37.9%, 22 out of 58) with high expression level, only two (9.5%) belonged to HA backgrounds (ST22-IVh, t032 and ST5-II, t002) whereas the majority (90.5%, n=19) were represented by CA backgrounds (Table 1 and Table 2). Moreover, two additional CA strains, ST72-IVa-t148 and ST8-MSSA-t008, showed expression rates near the cutoff value (5.73), with 5.76 and 5.87, respectively. These were considered as belonging to the low-level expression group.

The three strains with the highest expression rate were ST398-VII-t108 (2.85), ST398-IVa-t011 (3.25) and ST25-MSSA-t2909 (4.16) and strains with the lowest rate were ST1806 (TLV ST22)-IVh-t747 (9.28), ST45-IVa-t004 (10.38) and ST1-MSSA-t127 (11.09).

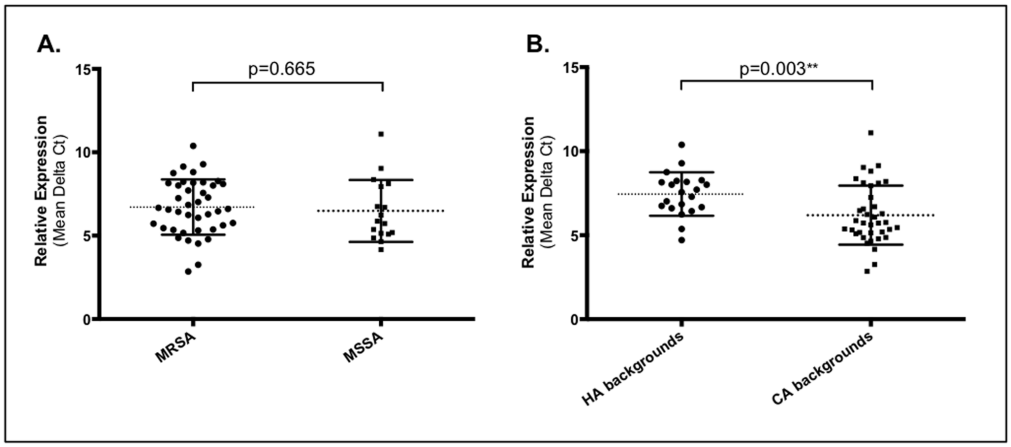


Figure 4. Distribution of the relative *hla* expression. Mean of relative expression of three independent readings. Expression comparison between a) MRSA and MSSA and b) HA and CA backgrounds using the Two-tailed Student's t-test. Statistically significance (p≤0.05) (**).

We observed that some promoters and gene alleles (P6, P12/P13, P21; and *hla7*, *hla9*, *hla11*) were exclusively associated to a high expression level profile, while others (P3/P4/P5, P7, P8/P9, P11, P15, P17/P18, P23; and *hla4*, *hla8*, *hla10*) were exclusively associated to a low expression level (Table 1 and 2). But we also found promoter and gene allotypes that were associated to both high and low expression levels.

DISCUSSION

Although Hla is one of the most important *S. aureus* virulence factors (5), to the best of our knowledge, this is the first study in which the variation in *hla* nucleotide sequence and gene expression was assessed in such a large and representative collection.

We found that the nucleotide sequence of *hla* was highly diverse. The high degree of diversity found within *hla* is in accordance to results obtained for other exotoxins, which are generally highly polymorphic (49). Four non-synonymous substitutions (Q87 stop codon, D208E, S239T and T243S) were identified, that are located in two structural protein domains which are essential for Hla oligomerization and pore formation (Rim and Cap)(29, 42, 48). The impact of these amino acid (aa) changes on *hla* activity is uncertain. If by one hand, the aa changes described implicate differences in the molecular weight of the aa, that can have influence in the three dimensional structure stability and activity of the protein; on the other hand these aa changes did not match any of the aa previously described to be essential for Hla pore formation.

Furthermore, Walker and Bayley showed that multiple mutations in this same region (residues spanning Hla235-250) did not alter Hla activity in terms of binding, oligomerization or lysis. Thus, it would not be expected that S239T or T243S had significant biological impact in terms of toxin function. The unique mutation with an identified role in Hla function is the stop codon found in the ST36 and ST30 strains that was previously described by DeLeo and co-authors (15) to hinder toxin production and to originate a less virulent strain in a murine infection model. The true effect of the non-synonymous substitutions identified in our study in the activity of the protein would have to be tested by the construction of site directed mutagenesis mutants and by performing binding, oligomerization, hemolysis and *in vivo* models assays.

The construction of phylogenetic trees from the *hla* defined the existence of two clusters with different levels of genetic diversity suggesting that *hla* is evolving at different rates in different genetic backgrounds. Interestingly, the most diverse cluster included the clonal types which are presently more disseminated or that emerged recently (like ST398). This might be related to the fact that these clones still need to evolve to evade the human immune system and not enough time as elapsed for the most adapted allele to have been selected (9). On the other hand the recombination events detected in the

hla gene in this study were all in strains belonging to the high genetic diversity cluster, suggesting that this mechanism might have been important in the most recent *hla* evolution and diversification.

Interestingly, the phylogenetic tree constructed from the *hla* gene was similar to that constructed from MLST genes, in the sense that both trees distributed the different STs similarly in two main clusters. This observation suggests that *hla* gene has evolved together with the *S. aureus* genetic background. A similar type of correlation with the genetic background was previously described for adhesins, either located in the core genome (*clfA*, *clfB*, *fnbA*, *map*, *sdrC*, and *spa*) or accessory genome (*ebpS*, *fnbB*, *sdrD*, and *sdrE*) (21). Although this was the case for the great majority of STs, we observed that four STs (ST22, ST121, ST59, ST93) were located in different clusters in the *hla* and MLST trees. Our results suggest that recombination occurring at the *hla* level, might explain the different clustering of strains belonging to ST22 and ST121. No recombination events were, however, detected in MLST genes or *hla* sequences of strains belonging to ST59 and ST93, suggesting that their displacement in the two trees could derive from different phenomena, like random mutation.

It was previously suggested that CA-MRSA expressed more *hla* than HA-MRSA (22). Results from our study allowed us to extend this conclusion to virtually all epidemic CA, but also in two particular cases of HA genetic backgrounds. The CA strains belonging to ST398, ST25, ST121 and ST93 showed uniformly high relative expression rates and strains belonging to ST36, ST45, ST239, ST247 and ST250 showed uniformly low expression rates. To understand if in fact these patterns of expression are characteristic of these clones, more strains within each clone should be studied for *hla* expression. Nevertheless, we could not correlate the *hla* expression rate with any particular polymorphism within the promoter or any aa substitution in the *hla* gene. The results suggest that *hla* regulation is probably a result of combination of factors which are redundant, rather than associated to a single genetic event. In fact, it has been demonstrated by several authors that alpha-hemolysin is part of a complex regulatory network, that includes the main two-component systems (TCS) – Agr – that in turn is controlled by a diverse pool of regulatory networks that coordinately interact in response to external stimulus and cell signals, namely others TCS (SaeRS, ArlRS and SrrAB), alternative sigma factors (σ^B), and transcription factors (e.g. SarS, SarT, Rot, SarA, SarZ) (31, 45).

We showed that *hla* evolved together with the genetic background. Moreover, the most epidemic CA-

MRSA genetic backgrounds express more *hla* than the most epidemic HA-MRSA genetic backgrounds. However, the finding of frequent strain-to-strain variation in the expression level of *hla* within strains of the same clonal types suggests that *hla* polymorphisms cannot be used as genetic markers of virulence and investigators should remain cautious when inferring conclusions for the entire MRSA population from studies performed with a limited number of strains.

ACKNOWLEDGMENTS

Partial support for this study was provided by project Ref. P-99911 from Fundação Calouste Gulbenkian and A. Tavares was supported by grants SFRH/BD/44220/2008 from Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

All the experimental work was done by A. Tavares

FUNDING

This study was funded by project Ref. P-99911 from Fundação Calouste Gulbenkian and additionally by FCT through grant Ref. Pest-OE/EQB/LAO004/2011, Portugal. A. Tavares was supported by grant SFRH/BD/44220/2008 from Fundação para a Ciência e Tecnologia, Lisbon, Portugal.

REFERENCES

1. **Aires-de-Sousa, M., B. Correia, H. de Lencastre, and C. Multilaboratory Project.** 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:**2912-7.
2. **Amorim, M. L., M. Aires de Sousa, I. S. Sanches, R. Sa-Leao, J. M. Cabeda, J. M. Amorim, and H. de Lencastre.** 2002. Clonal and antibiotic resistance profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) from a Portuguese hospital over time. Microb Drug Resist **8:**301-9.
3. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. 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.
4. **Ballal, A., B. Ray, and A. C. Manna.** 2009. sarZ, a sarA family gene, is transcriptionally activated by MgrA and is involved in the regulation of genes encoding exoproteins in *Staphylococcus aureus*. J Bacteriol **191:**1656-65.
5. **Berube, B. J., and J. Bubeck Wardenburg.** 2013. *Staphylococcus aureus* alpha-Toxin: Nearly a Century of Intrigue. Toxins (Basel) **5:**1140-66.
6. **Bubeck Wardenburg, J., T. Bae, M. Otto, F. R. Deleo, and O. Schneewind.** 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat Med **13:**1405-6.
7. **Bubeck Wardenburg, J., R. J. Patel, and O. Schneewind.** 2007. Surface proteins and exotoxins are required for the pathogenesis of *Staphylococcus aureus* pneumonia. Infect Immun **75:**1040-4.
8. **Burlak, C., C. H. Hammer, M. A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo.** 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. Cell Microbiol **9:**1172-90.
9. **Castillo-Ramirez, S., S. R. Harris, M. T. Holden, M. He, J. Parkhill, S. D. Bentley, and E. J. Feil.** 2011. The impact of recombination on dN/dS within recently emerged bacterial clones. PLoS Pathog **7:**e1002129.
10. **Conceicao, T., A. Tavares, M. Miragaia, K. Hyde, M. Aires-de-Sousa, and H. de Lencastre.** 2010. Prevalence and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Atlantic Azores islands: predominance of SCCmec types IV, V and VI. Eur J Clin Microbiol Infect Dis **29:**543-50.
11. **Crisostomo, 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.
12. **David, M. Z., and R. S. Daum.** Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev **23:**616-87.
13. **de Lencastre, H., M. Chung, and H. Westh.** 2000. Archaic strains of methicillin-resistant *Staphylococcus aureus*: molecular and microbiological properties of isolates from the 1960s in Denmark. Microb Drug Resist **6:**1-10.
14. **De'ath, G., and K. E. Fabricius.** 2000. CLASSIFICATION AND REGRESSION TREES: A POWERFUL YET SIMPLE TECHNIQUE FOR ECOLOGICAL DATA ANALYSIS. Ecology **81:**3178-3192.
15. **DeLeo, F. R., A. D. Kennedy, L. Chen, J. Bubeck Wardenburg, S. D. Kobayashi, B. Mathema, K. R. Braughton, A. R. Whitney, A. E. Villaruz, C. A. Martens, S. F. Porcella, M. J. McGavin, M. Otto, J. M. Musser, and B. N. Kreiswirth.** 2011. Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus*. Proc Natl Acad Sci U S A **108:**18091-6.
16. **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.
17. **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.

18. **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.
19. **Inoshima, I., N. Inoshima, G. A. Wilke, M. E. Powers, K. M. Frank, Y. Wang, and J. Bubeck Wardenburg.** 2011. A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nat Med **17:**1310-4.
20. **Kobayashi, S. D., J. M. Voyich, C. Burlak, and F. R. DeLeo.** 2005. Neutrophils in the innate immune response. Arch Immunol Ther Exp (Warsz) **53:**505-17.
21. **Kuhn, G., P. Francioli, and D. S. Blanc.** 2006. Evidence for clonal evolution among highly polymorphic genes in methicillin-resistant *Staphylococcus aureus*. J Bacteriol **188:**169-78.
22. **Li, M., G. Y. Cheung, J. Hu, D. Wang, H. S. Joo, F. R. Deleo, and M. Otto.** 2010. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J Infect Dis **202:**1866-76.
23. **Liang, X., J. W. Hall, J. Yang, M. Yan, K. Doll, R. Bey, and Y. Ji.** 2011. Identification of single nucleotide polymorphisms associated with hyperproduction of alpha-toxin in *Staphylococcus aureus*. PLoS One **6:**e18428.
24. **Liang, X., C. Yu, J. Sun, H. Liu, C. Landwehr, D. Holmes, and Y. Ji.** 2006. Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of *Staphylococcus aureus*. Infect Immun **74:**4655-65.
25. **Liang, X., L. Zheng, C. Landwehr, D. Lunsford, D. Holmes, and Y. Ji.** 2005. Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. J Bacteriol **187:**5486-92.
26. **Loughman, J. A., S. A. Fritz, G. A. Storch, and D. A. Hunstad.** 2009. Virulence gene expression in human community-acquired *Staphylococcus aureus* infection. J Infect Dis **199:**294-301.
27. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F. C. Tenover.** 2003. 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.
28. **Milheirico, 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*: 'SCCmec IV multiplex'. J Antimicrob Chemother **60:**42-8.
29. **Montoya, M., and E. Gouaux.** 2003. Beta-barrel membrane protein folding and structure viewed through the lens of alpha-hemolysin. Biochim Biophys Acta **1609:**19-27.
30. **Morfeldt, E., D. Taylor, A. von Gabain, and S. Arvidson.** 1995. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J **14:**4569-77.
31. **Novick, R. P.** 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol **48:**1429-49.
32. **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.
33. **Oliveira, D. C., C. Milheirico, S. Vinga, and H. de Lencastre.** 2006. Assessment of allelic variation in the ccrAB locus in methicillin-resistant *Staphylococcus aureus* clones. J Antimicrob Chemother **58:**23-30.
34. **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.
35. **Otto, M.** 2013. Community-associated MRSA: What makes them special? Int J Med Microbiol.
36. **Richardson, J. F., and S. Reith.** 1993. Characterization of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. J Hosp Infect **25:**45-52.

37. **Roberts, R. B., A. de Lencastre, W. Eisner, E. P. Severina, B. Shopsin, B. N. Kreiswirth, and A. Tomasz.** 1998. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in 12 New York hospitals. MRSA Collaborative Study Group. J Infect Dis **178:**164-71.
38. **Rolo, J., M. Miragaia, A. Turlej-Rogacka, J. Empel, O. Bouchami, N. A. Faria, A. Tavares, W. Hryniewicz, A. C. Fluit, H. de Lencastre, and C. W. Group.** 2012. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One **7:**e34768.
39. **Sa-Leao, R., I. Santos Sanches, D. Dias, I. Peres, R. M. Barros, and H. de Lencastre.** 1999. Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in international samples: relics of a formerly widely disseminated strain? J Clin Microbiol **37:**1913-20.
40. **Sanches, I. S., M. Ramirez, H. Troni, M. Abecassis, M. Padua, A. Tomasz, and H. de Lencastre.** 1995. Evidence for the geographic spread of a methicillin-resistant *Staphylococcus aureus* clone between Portugal and Spain. J Clin Microbiol **33:**1243-6.
41. **Schmidt, K. A., A. C. Manna, S. Gill, and A. L. Cheung.** 2001. SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. Infect Immun **69:**4749-58.
42. **Song, L., M. R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, and J. E. Gouaux.** 1996. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Science **274:**1859-66.
43. **Tamura, K., M. Nei, and S. Kumar.** 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci U S A **101:**11030-5.
44. **Tavares, A., M. Miragaia, J. Rolo, C. Coelho, H. de Lencastre, and C.-M. M. w. group.** 2013. High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* in the community in Portugal: evidence for the blurring of community-hospital boundaries. Eur J Clin Microbiol Infect Dis **32:**1269-83.
45. **Thoendel, M., J. S. Kavanaugh, C. E. Flack, and A. R. Horswill.** 2011. Peptide signaling in the staphylococci. Chem Rev **111:**117-51.
46. **Valeva, A., M. Palmer, and S. Bhakdi.** 1997. Staphylococcal alpha-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. Biochemistry **36:**13298-304.
47. **Vandenesch, F., J. Kornblum, and R. P. Novick.** 1991. A temporal signal, independent of agr, is required for hla but not spa transcription in *Staphylococcus aureus*. J Bacteriol **173:**6313-20.
48. **Walker, B., and H. Bayley.** 1995. Key residues for membrane binding, oligomerization, and pore forming activity of staphylococcal alpha-hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. J Biol Chem **270:**23065-71.
49. **Wilson, G. J., K. S. Seo, R. A. Cartwright, T. Connelley, O. N. Chuang-Smith, J. A. Merriman, C. M. Guinane, J. Y. Park, G. A. Bohach, P. M. Schlievert, W. I. Morrison, and J. R. Fitzgerald.** 2011. A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. PLoS Pathog **7:**e1002271.
50. **Zielinska, A. K., K. E. Beenken, H. S. Joo, L. N. Mrak, L. M. Griffin, T. T. Luong, C. Y. Lee, M. Otto, L. N. Shaw, and M. S. Smeltzer.** 2011. Defining the strain-dependent impact of the Staphylococcal accessory regulator (sarA) on the alpha-toxin phenotype of *Staphylococcus aureus*. J Bacteriol **193:**2948-58.

SUPPLEMENTARY DATA

Table S1. Molecular characterization of the 73 MRSA and MSSA strains included in this study.

Nº	Isolate ID	Year	Country (origin)	MRSA/ MSSA	SCC*mec* type	SCCmec IV subtype	PVL	spa type	ST	Clone final classification	Reference
1	HLZ6	2009	Portugal	MRSA	II	n.a.	-	t002	5	ST5	44
2	BK2464	1990	United Kindom	MRSA	II	n.a.	n.d.	t002	5	NY/JP	34, 37
3	HBR73	2006	Portugal	MRSA	II	n.a.	-	t067	5	NY/JP	1
4	C013	2002	Czech Republic	MRSA	VI	n.a.	+	t002	5	Pediatric	38
5	HDES26	2007-2008	Azores (PT)	MRSA	VI	n.a.	+	t062	5	Pediatric	10
6	HDE288	1996	Portugal	MRSA	VI	n.a.	-	t311	5	Pediatric	39
7	HSA29	1992-1993	Portugal	MSSA	n.a.	n.a.	n.d.	t002	5	ST5	2
8	HDE461	2006	Portugal	MRSA	IV	n.d.	-	t022	22	EMRSA15	1
9	HAR22	1991	United Kindom	MRSA	IV	IVh	-	t022	22	EMRSA15	28, 36
10	HSMB280	2009	Portugal	MRSA	IV	IVh	-	t032	22	EMRSA15	44
11	LBM12	2009	Portugal	MRSA	IV	IVh	-	t747	1806	ST1806 (TLV ST22)	44
12	HSMB184	2009	Portugal	MRSA	n.a.	n.a.	-	t5951	1806	ST1806 (TLV ST22)	44
13	HPH2	2006	Portugal	MRSA	II	n.a.	-	t018	36	EMRSA16	1
14	HAR24	1993	United Kindom	MRSA	II	n.a.	n.d.	t018	36	EMRSA16	33
15	DEN4415	2001	Denmark	MRSA	II	n.a.	n.d.	t021	36	EMRSA16	18
16	C563	2009	Denmark	MRSA	IV	IV_{NT}	-	t015	45	Berlin	38
17	C036	2004	Czech Republic	MRSA	V	NA	-	t015	45	Berlin	38
18	HAR38	1995	Belgium	MRSA	IV	IVa	-	t004	45	Berlin	33, 34
19	HFX77	2009	Portugal	MRSA	III	n.a.	-	t037	239	Brazilian	44
20	HUC343	2006	Portugal	MRSA	IIIA	n.a.	-	t037	239	Brazilian	1
21	HU25	1993	Brazil	MRSA	IIIA	n.a.	-	t138	239	Brazilian	34
22	BK1953	1995	United Kindom	MRSA	IA	n.a.	n.d.	t051	247	Iberian	33,37
23	HPV107	1992	Portugal	MRSA	IA	n.a.	n.d.	t051	247	Iberian	40
24	HSJ419	2006	Portugal	MRSA	IA	n.a.	-	t725	247	Iberian	1
25	E2125	1964	Denmark	MRSA	I	n.a.	n.d.	t051	247	Archaic	13
26	10395	1961	United Kindom	MRSA	I	n.a.	n.d.	t008	250	Archaic	11
27	COL	1965	United Kindom	MRSA	I	n.a.	n.d.	t008	250	Archaic	34
28	HFX74	2009	Portugal	MRSA	IV	IVa	+	t008	8	USA300	44
29	USA300	1995-2003	United States	MRSA	IV	IVa	+	t008	8	USA300	27
30	C438	2008	Sweden	MRSA	IV	IVc	+	t024	8	USA300	38
31	C574B	2009	Denmark	MRSA	IV	IVd	-	t1257	612	DLV ST8	38
32	LBM27	2009	Portugal	MSSA	n.a.	n.a.	-	t024	8	USA300 like	44
33	LBM74	2009	Portugal	MSSA	n.a.	n.a.	-	t008	8	ST8	44
34	C270	2009	Romania	MRSA	IV	IVa	-	t1381	1	USA400 like	38
35	USA400	1995-2003	United States	MRSA	IV	IVa	+	t127	1	USA400	27
36	LBM36	2009	Portugal	MSSA	n.a.	n.a.	-	t127	1	USA400 like	44
37	C577	2009	Denmark	MRSA	IV	IVa	+	t216	59	Taiwan	38
38	C583	2009	Denmark	MRSA	IV	IVa	-	t437	59	Taiwan	38
39	C434	2009	Sweden	MRSA	V	n.a.	+	t437	59	Taiwan	38
40	C018	2002	Czech Republic	MRSA	IV	IVa	+	t1819	93	Queensland	38
41	C491	no data	Netherlands	MRSA	IV	IVa	+	t202	93	Queensland	38
42	LBM54	2009	Portugal	MRSA	IV	n.d.	-	t011	398	ST398	44
43	C482	no data	Netherlands	MRSA	IV	IVa	-	t011	398	ST398	38
44	C496	no data	Netherlands	MRSA	VII	n.a.	-	t108	398	ST398	38
45	LBM40	2009	Portugal	MSSA	n.a.	n.a.	-	t034	398	ST398	44
46	C017	2004	Czech Republic	MRSA	IV	IVc	+	t019	30	Southwesth Pacific	38
47	C385	2005	Spain	MRSA	IV	IVc	+	t019	30	Southwesth Pacific	38
48	C479	2005	Netherlands	MRSA	IV	IVc	+	t019	30	Southwesth Pacific	38
49	HSJO7	2009	Portugal	MRSA	IV	n.d.	-	t148	72	USA700	44
50	USA700	1995-2003	United States	MRSA	IV	IVa	+	t148	72	USA700	27
51	C003	2003	Czech Republic	MRSA	IV	IVa	+	t791	72	USA700	38
52	SAMS1024	2009	Portugal	MRSA	IV	IVa	-	t1346	1810	ST1810 (SLV ST72)	44
53	HUC594	2009	Portugal	MSSA	n.a.	n.a.	-	t148	72	ST72	44
54	HFA28	2009	Portugal	MSSA	n.a.	n.a.	-	t126	72	ST72	44
55	C238	2008	Czech Republic	MSSA	n.a.	n.a.	-	t3682	72	ST72	38
56	C168	2005	Greece	MRSA	IV	IVc	+	t044	80	European	38
57	C485	no data	Netherlands	MRSA	IV	IVc	+	t044	80	European	38
58	C014	2002	Czech Republic	MRSA	IV	IVc	+	t131	80	European	38
59	LBM25	2009	Portugal	MSSA	n.a.	n.a.	-	t1509	15	ST15	44
60	C157	2009	United Kindom	MSSA	n.a.	n.a.	-	t084	15	ST15	38
61	C230	2009	Czech Republic	MSSA	n.a.	n.a.	-	t346	15	ST15	38
62	HBA33	2009	Portugal	MSSA	n.a.	n.a.	-	t258	25	ST25	44
63	C095	2005	Bulgaria	MSSA	n.a.	n.a.	+	t2909	25	ST25	38
64	C141	2009	United Kindom	MSSA	n.a.	n.a.	-	t081	25	ST25	38
65	HBA34	2009	Portugal	MRSA	IV	IV_{NT}	-	t308	121	ST121	44
66	HUC574	2009	Portugal	MSSA	n.a.	n.a.	+	t435	121	ST121	44
67	HUC587	2009	Portugal	MSSA	n.a.	n.a.	-	t159	121	ST121	44
68	HUC578	2009	Portugal	MSSA	n.a.	n.a.	+	t284	121	ST121	44
69	HFF204	2005-2006	Portugal	MSSA	n.a.	n.a.	+	t318	30	ST30	1
70	HFA30	2009	Portugal	MSSA	n.a.	n.a.	-	t012	30	ST30	44
71	HUC585	2009	Portugal	MSSA	n.a.	n.a.	-	t342	30	ST30	44
72	LBM23	2009	Portugal	MSSA	n.a.	n.a.	-	t100	9	ST9	44
73	HFX84	2009	Portugal	MSSA	n.a.	n.a.	-	t267	97	ST97	44

n.a. not apply; n.d. not determined; Y yes; N no; NT non typable; MSSA methicillin-susceptible *S. aureus*; MRSA methicillin-resistant *S. aureus*; (-) negative; (+) positive; TLV triple locus variant.

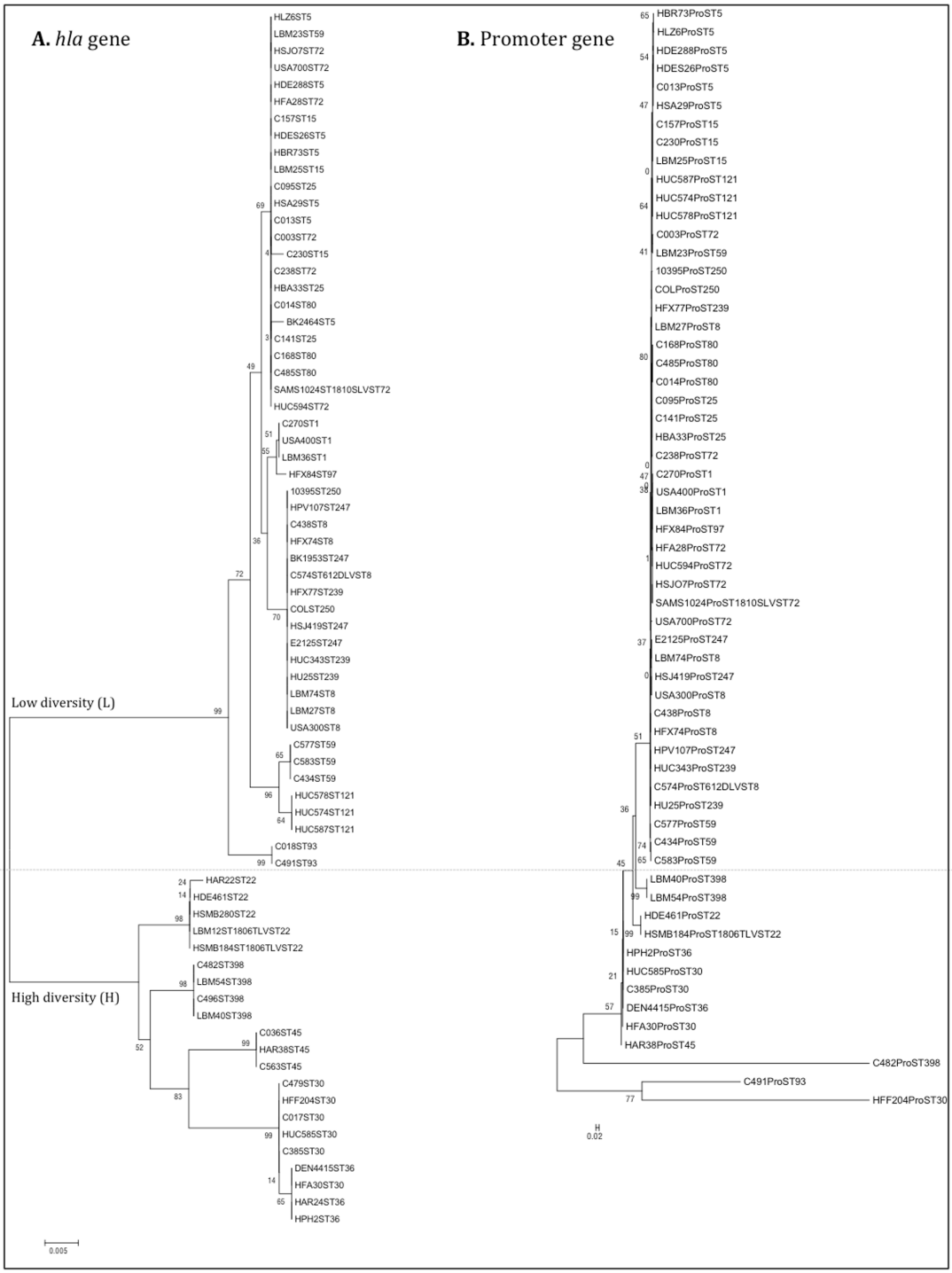


Figure S1. Phylogenetic trees of the *hla* gene and promoter gene. The tree was constructed using MEGA 5 with Neighbour-joining method and bootstrap values provided as percents over 1000 replications. Branch length values are indicated and the percentage of replicate trees (bootstrap test) are shown next to the branches.

		Time (min)							
		0	30	60	90	120	150	180	210
1	HLZ6C	0.182	0.222	0.344	0.540	0.759	0.932	1.062	1.173
2	HBR73C	0.267	0.324	0.500	0.697	0.887	1.066	1.190	1.282
3	BK2464C	0.259	0.310	0.462	0.621	0.800	0.982	1.115	1.220
4	C013C	0.237	0.277	0.429	0.641	0.856	1.041	1.182	1.298
5	HDES26C	0.261	0.308	0.466	0.678	0.903	1.052	1.173	1.259
6	HDE288C	0.257	0.305	0.445	0.643	0.842	1.022	1.171	1.269
7	HDE461C	0.245	0.268	0.357	0.517	0.686	0.866	1.031	1.158
8	HSMB280C	0.231	0.261	0.333	0.399	0.469	0.531	0.595	0.656
9	HAR22C	0.211	0.238	0.319	0.472	0.663	0.844	1.011	1.134
10	C563C	0.227	0.278	0.385	0.562	0.739	0.917	1.070	1.181
11	C036C	0.242	0.295	0.433	0.631	0.842	1.034	1.199	1.289
12	HAR38C	0.238	0.277	0.391	0.579	0.788	0.976	1.132	1.247
13	HFX77C	0.236	0.263	0.362	0.498	0.672	0.835	1.008	1.137
14	HUC343C	0.226	0.247	0.329	0.447	0.611	0.761	0.925	1.069
15	HU25C	0.241	0.268	0.360	0.494	0.658	0.826	0.988	1.120
16	HPV107C	0.217	0.238	0.317	0.484	0.680	0.898	1.078	1.217
17	E2125C	0.263	0.309	0.450	0.647	0.854	1.041	1.177	1.284
18	HFX74C	0.259	0.288	0.385	0.572	0.771	0.938	1.084	1.199
19	C438C	0.229	0.254	0.332	0.492	0.666	0.858	1.026	1.161
20	USA300C	0.223	0.257	0.352	0.541	0.760	0.953	1.104	1.221
21	C270C	0.262	0.320	0.502	0.718	0.941	1.078	1.175	1.263
22	C574BC	0.245	0.301	0.443	0.643	0.857	1.044	1.180	1.286
23	USA400C	0.226	0.261	0.386	0.592	0.815	1.025	1.177	1.289
24	C434C	0.247	0.285	0.396	0.593	0.805	0.993	1.145	1.265
25	C577C	0.256	0.309	0.430	0.626	0.833	1.016	1.151	1.278
26	C583C	0.262	0.309	0.439	0.654	0.859	1.030	1.159	1.266
27	C018C	0.265	0.326	0.491	0.699	0.899	1.070	1.202	1.295
28	C491C	0.262	0.313	0.468	0.690	0.891	1.052	1.179	1.275
29	LBM54C	0.259	0.309	0.466	0.675	0.870	1.035	1.163	1.252
30	C482C	0.255	0.297	0.445	0.647	0.825	0.982	1.109	1.198
31	C496C	0.225	0.278	0.413	0.630	0.829	0.986	1.121	1.228
32	C017C	0.229	0.262	0.382	0.567	0.749	0.883	1.015	1.131
33	C385C	0.225	0.257	0.357	0.522	0.649	0.793	0.914	1.023
34	C479C	0.235	0.268	0.372	0.565	0.769	0.945	1.090	1.212
35	HSJO7C	0.238	0.281	0.420	0.614	0.820	1.014	1.154	1.258
36	C003C	0.235	0.273	0.389	0.596	0.771	0.908	1.025	1.144
37	USA700C	0.240	0.280	0.414	0.619	0.841	1.016	1.137	1.235
38	C014C	0.257	0.306	0.477	0.686	0.903	1.069	1.184	1.271
39	C168C	0.246	0.289	0.449	0.665	0.889	1.082	1.208	1.290
40	C485C	0.243	0.282	0.434	0.648	0.863	1.050	1.197	1.285
41	HBA34C	0.229	0.275	0.399	0.607	0.828	1.002	1.137	1.236
42	LBM12C	0.229	0.265	0.386	0.591	0.756	0.935	1.056	1.154
43	LBM25C	0.232	0.270	0.407	0.609	0.829	1.048	1.194	1.295
44	C157C	0.239	0.277	0.416	0.607	0.822	1.021	1.174	1.279
45	C230C	0.236	0.273	0.384	0.567	0.771	0.976	1.142	1.252
46	C095C	0.226	0.268	0.394	0.595	0.745	0.910	1.062	1.202
47	HUC574C	0.231	0.267	0.363	0.540	0.745	0.913	1.069	1.185
48	HUC587C	0.215	0.241	0.332	0.511	0.704	0.913	1.073	1.205
49	HUC578C	0.225	0.258	0.376	0.584	0.812	1.008	1.166	1.270
50	HFF204C	0.228	0.265	0.403	0.605	0.809	0.971	1.108	1.222
51	HFA30C	0.238	0.303	0.469	0.646	0.824	0.965	1.109	1.219
52	HUC585C	0.226	0.287	0.447	0.611	0.762	0.894	1.031	1.146
53	HFA28C	0.232	0.301	0.466	0.680	0.892	1.075	1.201	1.292
54	C238C	0.233	0.297	0.458	0.667	0.870	1.046	1.169	1.259
55	LBM23C	0.227	0.294	0.473	0.672	0.824	0.967	1.084	1.180
56	HFX84C	0.240	0.309	0.486	0.724	0.941	1.125	1.232	1.311
57	LBM36C	0.216	0.270	0.419	0.613	0.801	0.964	1.105	1.207
58	LBM27C	0.222	0.282	0.410	0.608	0.799	0.983	1.126	1.237
59	LBM74C	0.226	0.286	0.431	0.625	0.813	0.972	1.098	1.202
60	LBM40C	0.213	0.268	0.388	0.580	0.751	0.895	1.029	1.144
61	HSA29C	0.230	0.293	0.458	0.659	0.861	1.038	1.177	1.267
62	HPH2C	0.223	0.275	0.388	0.546	0.676	0.823	0.941	1.077
63	DEN4415C	0.206	0.246	0.332	0.479	0.611	0.768	0.923	1.063
64	HAR24C	0.188	0.213	0.271	0.376	0.505	0.625	0.760	0.900
65	HSJ419C	0.216	0.255	0.344	0.503	0.704	0.910	1.088	1.229
66	BK1953C	0.206	0.233	0.295	0.388	0.539	0.690	0.870	1.031
67	10395C	0.215	0.261	0.361	0.535	0.710	0.862	0.987	1.101
68	COLC	0.200	0.218	0.259	0.310	0.379	0.474	0.554	0.666
69	SAMS1024C	0.222	0.268	0.384	0.567	0.743	0.878	1.010	1.116
70	HBA33C	0.215	0.270	0.399	0.598	0.784	0.942	1.080	1.208
71	C141C	0.206	0.261	0.397	0.591	0.754	0.916	1.061	1.188
72	HUC594C	0.218	0.272	0.394	0.573	0.729	0.881	1.019	1.128
73	HSMB184C	0.219	0.272	0.384	0.564	0.722	0.880	1.017	1.140

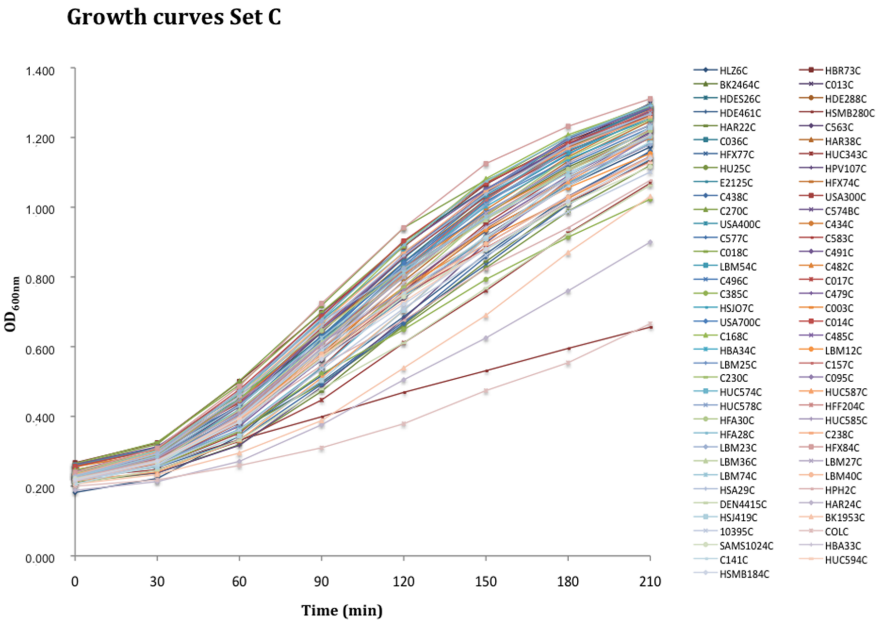


Figure S2.I. Growth curves for triplicates of each *S. aureus* strain – Set C

		Time (min)							
		0	30	60	90	120	150	180	210
1	HLZ6D	0.188	0.227	0.346	0.543	0.756	0.934	1.066	1.178
2	HBR73D	0.279	0.336	0.518	0.708	0.903	1.091	1.225	1.316
3	BK2464D	0.264	0.312	0.468	0.622	0.809	0.995	1.136	1.243
4	C013D	0.241	0.279	0.428	0.638	0.860	1.047	1.201	1.315
5	HDES26D	0.263	0.308	0.469	0.680	0.907	1.065	1.185	1.268
6	HDE288D	0.254	0.298	0.441	0.628	0.825	1.003	1.150	1.255
7	HDE461D	0.244	0.267	0.356	0.520	0.692	0.877	1.037	1.166
8	HSMB280D	0.237	0.261	0.333	0.399	0.464	0.526	0.583	0.643
9	HAR22D	0.213	0.237	0.319	0.473	0.665	0.852	1.004	1.164
10	C563D	0.232	0.282	0.393	0.579	0.758	0.948	1.111	1.228
11	C036D	0.252	0.302	0.442	0.641	0.857	1.052	1.213	1.304
12	HAR38D	0.239	0.275	0.395	0.585	0.797	0.991	1.133	1.258
13	HFX77D	0.236	0.262	0.363	0.494	0.672	0.839	0.997	1.131
14	HUC343D	0.224	0.248	0.327	0.438	0.603	0.759	0.917	1.059
15	HU25D	0.24	0.271	0.361	0.492	0.647	0.811	0.976	1.106
16	HPV107D	0.219	0.240	0.316	0.479	0.672	0.882	1.073	1.221
17	C2125D	0.256	0.297	0.435	0.629	0.834	1.026	1.161	1.270
18	HFX74D	0.261	0.288	0.385	0.575	0.785	0.955	1.099	1.210
19	C438D	0.225	0.250	0.325	0.480	0.676	0.864	1.027	1.160
20	USA300D	0.227	0.261	0.366	0.564	0.785	0.978	1.130	1.241
21	C270D	0.259	0.307	0.466	0.676	0.906	1.050	1.151	1.232
22	C574BD	0.250	0.305	0.449	0.650	0.869	1.051	1.192	1.292
23	USA400D	0.226	0.257	0.377	0.590	0.803	1.013	1.162	1.271
24	C434D	0.245	0.283	0.393	0.587	0.800	0.977	1.126	1.239
25	C577D	0.255	0.309	0.433	0.635	0.848	1.026	1.161	1.254
26	C583D	0.258	0.306	0.439	0.652	0.864	1.032	1.161	1.260
27	C018D	0.262	0.317	0.484	0.698	0.904	1.071	1.209	1.297
28	C491D	0.261	0.308	0.470	0.699	0.903	1.068	1.198	1.289
29	LBM54D	0.260	0.309	0.470	0.678	0.879	1.041	1.168	1.249
30	C482D	0.264	0.312	0.470	0.679	0.871	1.029	1.157	1.248
31	C496D	0.234	0.284	0.416	0.628	0.833	0.988	1.120	1.228
32	C017D	0.235	0.273	0.397	0.594	0.780	0.923	1.046	1.159
33	C385D	0.223	0.247	0.341	0.511	0.628	0.774	0.897	0.995
34	C479D	0.239	0.271	0.384	0.573	0.773	0.952	1.102	1.227
35	HSJO7D	0.240	0.283	0.430	0.629	0.840	1.036	1.172	1.280
36	C003D	0.234	0.274	0.392	0.599	0.778	0.926	1.039	1.140
37	USA700D	0.240	0.280	0.409	0.622	0.847	1.036	1.170	1.263
38	C014D	0.251	0.297	0.465	0.676	0.890	1.062	1.181	1.265
39	C168D	0.242	0.287	0.446	0.661	0.879	1.062	1.192	1.283
40	C485D	0.245	0.287	0.447	0.665	0.877	1.054	1.206	1.295
41	HBA34D	0.224	0.270	0.396	0.595	0.811	0.973	1.106	1.201
42	LBM12D	0.222	0.257	0.381	0.585	0.751	0.923	1.035	1.127
43	LBM25D	0.226	0.268	0.393	0.585	0.794	1.001	1.146	1.251
44	C157D	0.239	0.277	0.419	0.620	0.820	1.017	1.160	1.255
45	C230D	0.231	0.271	0.392	0.568	0.764	0.953	1.111	1.207
46	C095D	0.222	0.268	0.395	0.589	0.745	0.899	1.041	1.162
47	HUC574D	0.221	0.258	0.360	0.533	0.739	0.915	1.053	1.163
48	HUC587D	0.214	0.237	0.330	0.504	0.697	0.893	1.055	1.188
49	HUC578D	0.221	0.259	0.373	0.577	0.798	0.981	1.132	1.234
50	HFF204D	0.226	0.268	0.409	0.616	0.808	0.959	1.103	1.218
51	HFA30D	0.238	0.300	0.465	0.639	0.825	0.973	1.118	1.241
52	HUC585D	0.231	0.290	0.448	0.609	0.775	0.907	1.052	1.181
53	HFA28D	0.237	0.307	0.465	0.681	0.902	1.089	1.223	1.312
54	C238D	0.235	0.300	0.452	0.663	0.882	1.066	1.195	1.281
55	LBM23D	0.230	0.298	0.474	0.678	0.836	0.989	1.108	1.198
56	HFX84D	0.241	0.311	0.477	0.722	0.943	1.132	1.247	1.323
57	LBM36D	0.216	0.268	0.406	0.606	0.798	0.959	1.094	1.196
58	LBM27D	0.221	0.280	0.402	0.601	0.798	0.986	1.125	1.231
59	LBM74D	0.225	0.281	0.429	0.624	0.816	0.972	1.102	1.190
60	LBM40D	0.220	0.273	0.408	0.598	0.770	0.917	1.052	1.164
61	HSA29D	0.229	0.289	0.441	0.640	0.847	1.025	1.161	1.255
62	HPH2D	0.223	0.265	0.377	0.541	0.658	0.807	0.923	1.061
63	DEN4415D	0.209	0.248	0.335	0.482	0.619	0.766	0.927	1.062
64	HAR24D	0.197	0.222	0.285	0.394	0.515	0.637	0.781	0.926
65	HSJ419D	0.219	0.259	0.351	0.507	0.689	0.874	1.026	1.155
66	BK1953D	0.202	0.232	0.293	0.383	0.525	0.672	0.831	0.972
67	10395D	0.212	0.257	0.362	0.536	0.701	0.846	0.976	1.084
68	COLD	0.199	0.217	0.260	0.314	0.380	0.470	0.556	0.656
69	SAMS1024D	0.216	0.263	0.381	0.570	0.732	0.866	0.979	1.071
70	HBA33D	0.216	0.271	0.409	0.614	0.789	0.937	1.062	1.172
71	C141D	0.208	0.268	0.406	0.602	0.769	0.931	1.064	1.173
72	HUC594D	0.212	0.259	0.375	0.512	0.627	0.761	0.891	1.029
73	HSMB184D	0.214	0.260	0.378	0.505	0.621	0.764	0.898	1.022

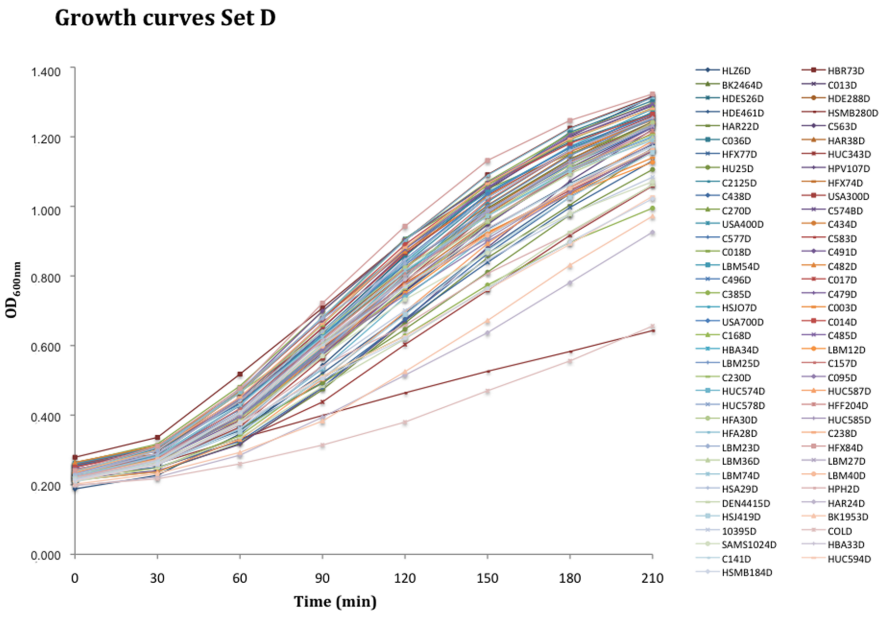


Figure S2.II. Growth curves for triplicates of each *S. aureus* strain – Set D.

		Time (min)							
		0	30	60	90	120	150	180	210
1	HLZ6E	0.250	0.327	0.498	0.705	0.883	1.026	1.119	1.209
2	HBR73E	0.234	0.310	0.486	0.677	0.872	1.040	1.145	1.245
3	BK2464E	0.250	0.313	0.472	0.630	0.816	0.971	1.096	1.198
4	C013E	0.224	0.291	0.457	0.668	0.878	1.050	1.179	1.277
5	HDES26E	0.253	0.319	0.491	0.701	0.910	1.023	1.150	1.208
6	HDE288E	0.237	0.307	0.460	0.653	0.848	1.008	1.140	1.228
7	HDE461E	0.217	0.252	0.353	0.527	0.704	0.885	1.033	1.154
8	HSMB280E	0.208	0.246	0.314	0.385	0.461	0.503	0.575	0.621
9	HAR22E	0.216	0.264	0.378	0.555	0.759	0.936	1.070	1.166
10	C563E	0.215	0.270	0.395	0.560	0.745	0.928	1.060	1.158
11	C036E	0.234	0.292	0.456	0.670	0.886	1.074	1.193	1.283
12	HAR38E	0.237	0.307	0.449	0.641	0.840	1.020	1.125	1.233
13	HFX77E	0.211	0.250	0.334	0.451	0.609	0.750	0.894	1.031
14	HUC343E	0.209	0.242	0.322	0.442	0.593	0.733	0.874	1.006
15	HU25E	0.213	0.244	0.326	0.448	0.598	0.744	0.868	1.018
16	HPV107E	0.210	0.247	0.33	0.508	0.704	0.897	1.090	1.195
17	E2125E	0.230	0.291	0.431	0.627	0.841	0.981	1.127	1.210
18	HFX74E	no data	no data	no data	no data	no data	no data	no data	no data
19	C438E	0.323	0.386	0.507	0.683	0.869	1.010	1.144	1.229
20	USA300E	0.217	0.265	0.373	0.573	0.798	0.974	1.114	1.191
21	C270E	0.230	0.291	0.453	0.637	0.852	1.001	1.110	1.183
22	C574BE	0.225	0.286	0.437	0.643	0.841	1.022	1.133	1.232
23	USA400E	0.212	0.262	0.376	0.561	0.792	0.979	1.119	1.219
24	C434E	0.218	0.271	0.406	0.606	0.812	0.990	1.111	1.218
25	C577E	0.222	0.274	0.393	0.570	0.766	0.953	1.080	1.180
26	C583E	0.222	0.278	0.401	0.601	0.816	0.975	1.091	1.179
27	C018E	0.217	0.280	0.426	0.629	0.845	0.994	1.127	1.209
28	C491E	0.229	0.293	0.450	0.656	0.857	1.023	1.142	1.229
29	LBM54E	0.236	0.306	0.477	0.687	0.868	1.014	1.133	1.210
30	C482E	0.234	0.294	0.451	0.645	0.837	0.988	1.096	1.171
31	C496E	0.225	0.288	0.444	0.658	0.858	1.012	1.129	1.221
32	C017E	0.213	0.264	0.383	0.575	0.782	0.967	1.102	1.209
33	C385E	0.224	0.280	0.409	0.544	0.711	0.831	0.945	1.015
34	C479E	0.218	0.268	0.372	0.540	0.758	0.936	1.078	1.182
35	HSJO7E	0.218	0.269	0.406	0.607	0.847	1.028	1.158	1.244
36	C003E	0.209	0.263	0.396	0.573	0.758	0.911	1.031	1.114
37	USA700E	0.172	0.213	0.314	0.521	0.735	0.922	1.074	1.173
38	C014E	0.236	0.311	0.486	0.698	0.899	1.053	1.171	1.240
39	C168E	0.231	0.298	0.473	0.694	0.912	1.095	1.211	1.276
40	C485E	0.238	0.300	0.464	0.675	0.892	1.078	1.207	1.278
41	HBA34E	0.241	0.307	0.455	0.681	0.891	1.070	1.187	1.273
42	LBM12E	0.228	0.288	0.438	0.612	0.808	0.966	1.072	1.133
43	LBM25E	0.233	0.303	0.456	0.670	0.897	1.105	1.239	1.320
44	C157E	0.242	0.312	0.482	0.695	0.902	1.084	1.209	1.281
45	C230E	0.220	0.264	0.372	0.550	0.751	0.936	1.100	1.213
46	C095E	0.228	0.293	0.444	0.618	0.789	0.928	1.067	1.192
47	HUC574E	0.227	0.287	0.400	0.583	0.784	0.954	1.095	1.187
48	HUC587E	0.215	0.253	0.349	0.529	0.728	0.939	1.099	1.220
49	HUC578E	0.232	0.285	0.411	0.609	0.841	1.038	1.182	1.260
50	HFF204E	0.226	0.283	0.426	0.622	0.831	1.001	1.124	1.222
51	HFA30E	0.229	0.295	0.463	0.631	0.798	0.947	1.073	1.199
52	HUC585E	0.227	0.286	0.446	0.605	0.751	0.899	1.020	1.140
53	HFA28E	0.228	0.300	0.464	0.666	0.868	1.060	1.177	1.283
54	C238E	0.218	0.279	0.427	0.631	0.834	1.022	1.143	1.242
55	LBM23E	0.231	0.304	0.487	0.682	0.826	0.972	1.072	1.175
56	HFX84E	0.233	0.303	0.481	0.703	0.911	1.110	1.215	1.296
57	LBM36E	0.214	0.271	0.421	0.615	0.810	0.984	1.110	1.214
58	LBM27E	0.205	0.264	0.378	0.590	0.780	0.964	1.094	1.204
59	LBM74E	0.224	0.286	0.434	0.618	0.818	0.953	1.089	1.193
60	LBM40E	0.216	0.273	0.404	0.597	0.756	0.909	1.036	1.146
61	HSA29E	0.222	0.285	0.445	0.639	0.846	1.021	1.137	1.237
62	HPH2E	0.208	0.246	0.335	0.482	0.601	0.745	0.863	1.016
63	DEN4415E	0.203	0.237	0.304	0.424	0.549	0.690	0.828	0.957
64	HAR24E	0.196	0.224	0.284	0.391	0.497	0.624	0.760	0.897
65	HSJ419E	0.197	0.232	0.314	0.459	0.640	0.832	1.011	1.161
66	BK1953E	0.192	0.217	0.272	0.355	0.502	0.658	0.831	0.983
67	10395E	0.207	0.250	0.341	0.506	0.641	0.794	0.960	1.107
68	COLE	0.195	0.210	0.244	0.296	0.359	0.439	0.521	0.606
69	SAMS1024E	0.211	0.260	0.375	0.568	0.736	0.873	0.993	1.095
70	HBA33E	0.210	0.269	0.411	0.612	0.788	0.942	1.077	1.181
71	C141E	0.181	0.236	0.365	0.557	0.718	0.905	1.053	1.182
72	HUC594E	0.196	0.245	0.362	0.534	0.680	0.838	0.977	1.083
73	HSMB184E	0.210	0.264	0.386	0.520	0.631	0.769	0.893	1.006

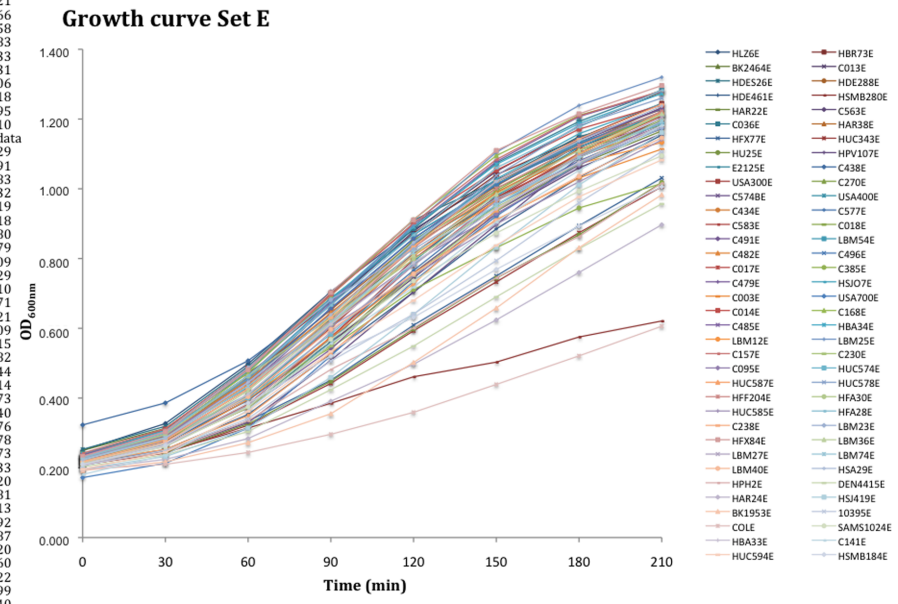


Figure S2.III. Growth curves for triplicates of each *S. aureus* strain – Set E.

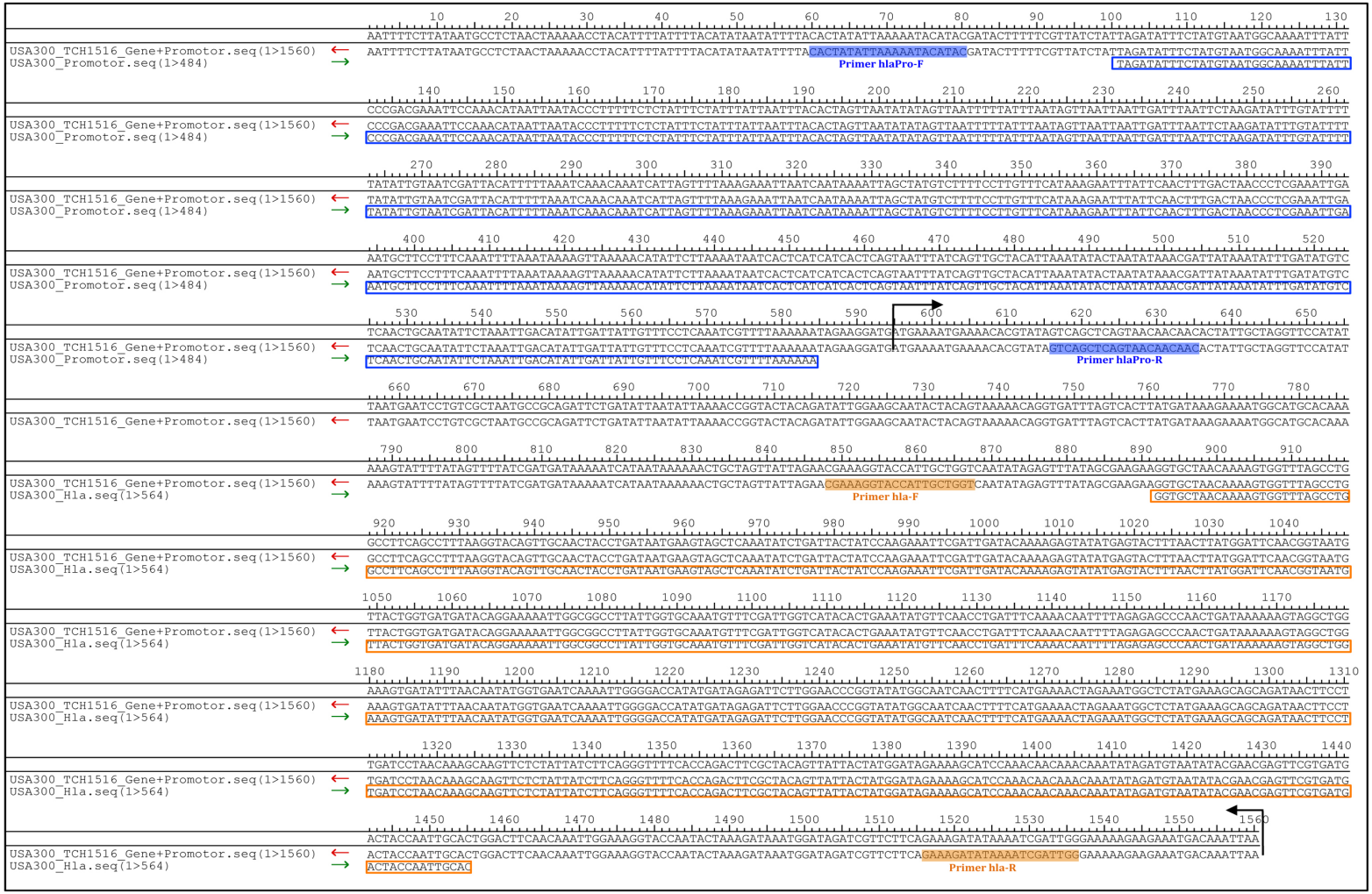


Figure S3. Internal sequences of *hla* promoter (highlighted blue) and *hla* gene (highlighted orange) used for analysis in this study. Primers used are highlighted. The sequence shown corresponds to the promoter and *hla* regions of USA300 strain from our collection blasted against USA300_TCH1516.

CHAPTER V

Concluding Remarks

CHAPTER V

Concluding Remarks

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been for over 30 years one of the most successful opportunistic pathogens in hospitals; however in the last two decades there has been an increasing recognition of MRSA as an agent of severe infection in the community. The success of MRSA is associated with its virulence and remarkable capacity to accumulate antimicrobial resistance determinants.

According to EARS-Net, Portugal has been placed for several years in the ranking of the European countries with the highest MRSA prevalence in hospitals (HA-MRSA) and in 2012, Portugal was the European country with the second highest MRSA prevalence in nosocomial invasive infections, reaching 53.8%. Due to the huge burden of MRSA infections in hospitals, the national MRSA surveillance performed so far had been mostly limited to MRSA, and to the hospital setting with little coverage of MSSA and MRSA with community origin (CA-MRSA).

Despite the increasing number of reports of CA-MRSA infections worldwide, in Portugal, only sporadic descriptions of infections episodes caused by CA-MRSA have appeared in the literature (10, 44, 49). In this Thesis we shed light on the clinical relevance of CA-MRSA in Portugal, elucidating for the first time the CA-MRSA prevalence and population structure.

To understand the possible origin of MRSA found in the community in Portugal, and how the emergence of MRSA in the community might have shaped the MSSA population, we analyzed the MSSA population dynamics and geographical distribution over almost two decades in the community and hospital.

Moreover, to understand if the enhanced virulence of CA-MRSA is correlated with particular alpha-hemolysin (*hla*) alleles or patterns of expression, we evaluated nucleotide sequence variation and gene expression profiles in a representative collection. The results obtained contributed to the elucidation of the alpha-hemolysin evolutionary history and the understanding of expression patterns among both CA and HA-MRSA.

MRSA in the community in Portugal: low prevalence of CA-MRSA and dominance of HA-MRSA clones

Several lines of evidence suggest that CA-MRSA are emergent pathogens in Europe. The data described in Chapter II provides the first insights into the prevalence, population structure and origin of CA-MRSA in the whole Portugal, resulting from the molecular characterization of MRSA and MSSA with community origin, mainly from infection (different clinical products).

Our data indicates that MRSA is present in a high frequency in the community (21.6%). This value is much higher than what was found in past studies among nasopharynx colonization in children and nasal colonization in draftees (<1%) (54, 59), among colonization in outpatients (5.1%) (3), healthcare workers (4.8%) (3) and in pediatric SSTIs (7.9%) (10). Such differences in frequency, between our study and the previous studies, probably reflect differences in the populations enrolled and screened and the type of samples analyzed.

Unexpectedly, among the MRSA found in the community in Portugal, only a small proportion was represented by CA-MRSA genetic backgrounds (11.4%). The prevalence of CA-MRSA in infections vary in different European countries with CA-MRSA being more relevant in the northern European countries as Denmark (29%) and Sweden (56%) (18, 36), in opposition to relatively low frequencies observed in the other European countries, such as Spain (1.5%) (38), Italy (6%) (39), Germany (14%) (29), France (18%) (62) and Greece (30%) (65). Moreover, this scenario is in clear contrast to what is observed in the USA (>50%) (43) and the United Arabic Emirates (73.1%) (58) where CA-MRSA is a significant cause of infections in the community. The variation of CA-MRSA population structures in different countries probably results from different social-cultural habits, healthcare policies, migratory fluxes, tourist and business travels that may influence the dynamics of clone dissemination.

Notably, the CA-MRSA population in Portugal was represented by diverse genetic backgrounds, including the pandemic clones USA300, USA400, USA700, European, Southwest Pacific (SWP), ST398 and the less disseminated ST1810 (SLV of ST72).

Some of these and other CA-MRSA genetic backgrounds were previously reported in Portugal, in a few studies in the community (56) but also in hospitals (1).

This high genetic diversity found in Portugal seems to be common to what was shown in other European countries (53). The exact reason for this scenario in opposition to the monoclonal predominance of USA300 observed in the USA is still a lively debate. The extraordinary

evolutionary success of USA300 clone found in the USA, has been attributed to the presence of specific virulence factors such as presence of arginine catabolic mobile element (ACME), the spermidine acetyltransferase (*sepG*), Panton-Valentine leukocidin (PVL) and higher expression of virulence factors, providing USA300 clone with a major selective advantage during colonization and infection (30, 48, 60). The USA300 clone found in our studies, and all over Europe, do not carry all the characteristic features of the USA300 prototype clone (53), which might explain its lack of selective advantage and success in the community, and also in hospitals. Moreover, the finding in the community of clonal types of the same genetic background as MSSA and MRSA with and without PVL may suggest local emergence of CA-MRSA clones.

An unexpected finding of our studies was the observation that a high proportion of the MRSA present in the community belonged to typical HA-MRSA epidemic clones (88.6%), in particular to the most prevalent clone found nowadays in Portuguese hospitals, the EMRSA-15 (77.2%), but also the NY/JP clone (14.9%). This is worrisome, since it shows the extensive dissemination of isolates from the hospital into the community. The same scenario has been reported in other countries, where the major nosocomial MRSA were described in the community. In particular, in France, the main clone found in the hospitals, the Lyon clone, corresponded to 80.6% of MRSA isolates found in the community (40). Also, in Spain, the most widespread clone in the hospitals, the ST125-IV, corresponded to 58.6% of MRSA isolates found in the community (23). The spread of the HA-MRSA clones ST5-II, t002 and ST239-III, t037 into the community was also observed in some Asian countries including Korea, Taiwan, Thailand, Vietnam and Sri Lanka (57). Several reports of EMRSA-15 in the community were registered in Italy (39) and England (27). Notwithstanding, this behavior seems to be specific of certain MRSA clones, which have the ability to survive and spread in both environments, as reviewed in Chapter II.

The EMRSA-15 clone has been reported by others to accumulate typical CA-MRSA genetic features, namely the presence of PVL (14), ACME (55) and high virulence gene expression (8), which allows this clone to become very well adapted to the community. In addition, the carriage of the small cassette SCC*mec* IV, associated to a lower fitness cost, has been described to be key to the success of EMRSA-15 in the community (28, 31, 32). Although it has been speculated that the SCC*mec* IV element in EMRSA-15 is less burdensome than larger size cassettes, recently Knight and colleagues found no evidence of a fitness cost attributed to the carriage of SCC*mec* II in CC30

in comparison to carriage to SCC*mec* IV in CC22 (31). The authors found that lineage rather than the size of SCC*mec* was the most important determinant of fitness. We may speculate that what we are observing in the community in Portugal (and other countries) may reflect this "lineage background theory", where not only EMRSA-15, but also the NY/JP clone, after hospital escape, seem to be well adapted to the community, in coexistence and for a long time.

In the future, it would be interesting to perform competition assays between MRSA strains belonging to these clones, and their MSSA counterparts, in order to understand possible differences in fitness, and their correlation with background or SCC*mec* size cassettes.

The clear evidence of blurring of hospital-community boundaries, strongly emphasize the imperative need to apply strict infection control measures in Portuguese hospitals. We think that "search and destroy" policies against MRSA as the ones implemented in the Northern European countries (33, 63) may not be possible to apply in Portugal, since we have, in opposition to these countries, high prevalence of MRSA in hospitals, and highly endemic clones, rendering this approach not sustainable. In our opinion, hospital infection control measures should focus first in the implementation of antimicrobial prescription policies (also in healthcare centers) directed to the predominant EMRSA-15 clone.

The emergence of EMRSA-15 in the UK has occurred at the same time as the introduction of ciprofloxacin (fluoroquinolone) into clinical practice in this country, which is believed to have triggered the dissemination of ciprofloxacin resistant EMRSA-15 clone in detriment of other clones (26). Data suggests that the EMRSA-15 clone probably found the ideal conditions to spread in Portugal, where fluoroquinolones are the second most used class of antibiotics (10.1%) (13). The extensive use of the quinolones in clinical practice in Portugal is not exclusive to hospitals but is also observed in the outpatient setting, as reported by the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) (15). In 2008, Portugal showed, in the ambulatory care, high rates of use of penicillins and cephalosporin (51%), followed by macrolides, lincosamides and streptogramins (17%) and quinolones (14%)(50). Among the quinolones, ciprofloxacin and levofloxacin were the most frequently used antibiotics in the community.

We believe that by following the actual national antimicrobial policies, we are perpetuating the long stay of the EMRSA-15 clone in Portugal both in the hospital and in the community. Some studies have shown that the implementation of a fluoroquinolone control program decrease significantly the

MRSA rates in the hospital setting. In a tertiary university hospital located in France, a study was undertaken to determine whether a hospital wide increase in fluoroquinolone use would be followed by an increase in MRSA (47). With that propose, a 10-years interrupted time-series analysis was implemented, accordingly: i) one-year period of 90% restriction on the use of all fluoroquinolones (January 2001 to January 2002) ii) two years of fluoroquinolones reintroduction increased up to prerestriction levels (January 2002 to December 2004) and iii) no intervention on fluoroquinolone use (an observational period) (January 2005 to June 2009). The authors observed a temporal association between fluoroquinolone use and MRSA trends, with a significant increase in MRSA after the reintroduction of fluoroquinolone. Moreover, the implementation of a fluoroquinolone control programme in the Hospital Saint-Louis (Paris, France) significantly decreased the MRSA rates from 27% to 21%, over a 4 year-period (35). Similarly, the implementation of such a program might help to decrease the rates of MRSA, in particular the EMRSA-15, in the hospitals in Portugal, and consequently in the community. Finally, we think that, the continuous follow-up of how EMRSA-15 resistance develops is also an important strategy of infection control that can help to adjust antimicrobial prescription policies.

The overwhelming representation of HA-MRSA clones in the community indicates that these clones are escaping from hospitals. We believe that the second control strategy should be focused on controlling the spread between these two environments. The most probable MRSA means of dissemination from hospitals into the community is hand-to-hand and hand-environment contact. For this reason, the improvement of hospital hygiene measures would certainly contribute to the decrease of the nosocomial MRSA prevalence, and the subsequent spread into the community. This include hand hygiene, of health-care workers, patients, visitors, as well as surfaces cleaning, restriction in the use of healthcare workers clothing and decolonization of colonized patients/healthcare workers. Information campaigns about the importance of the adequate hand hygiene should also be implemented in entrance-exit of hospitals and health care facilities, through advertisement divulgation, complemented with the broad distribution of hand-wash containers. Following good hospital practices, the MRSA burden in the community may decrease from 21.6% (including both HA-MRSA and CA-MRSA backgrounds) to 2.6% (associated exclusively to CA-MRSA backgrounds). We believe that the continuation of this type of surveillance studies is crucial to follow up of the evolution of MRSA.

Moreover, campaigns on the community alerting for the "Portuguese MRSA reality" and focused on the awareness for the importance of individual hand hygiene, complemented with the broad distribution (and maintenance) of hand washing devices (similar to what was done in the avian influenza H5N1 pandemic campaign), e.g. healthcare intitutions, nursing home, schools, public transports, public services, could help to control MRSA in the community. Moreover, the establishment of standard protocols for environmental decontamination in public services should take place.

MSSA population structure in the community in Portugal

Despite the presence of MRSA in the community, MSSA showed higher prevalence (78.4%) and a higher genetic diversity when compared to MRSA. Similarly to MRSA, MSSA were represented by both isolates related with CA (50.8%) and HA genetic backgrounds (49.2%). Almost half of CA-MSSA (46.7%) was related with the CA-MRSA epidemic clones, namely USA700, USA400, USA300, ST398, SWP and Taiwan clones. Concerning HA-MSSA the great majority (92.6%) were related with HA-MRSA epidemic clones, namely EMRSA-16, New/York (NY/JP), Berlin, EMRSA-15, and the Pediatric clone. The results obtained suggest that the MSSA and MRSA populations in the community are highly similar, suggesting that, in this environment, SCC*mec* can be frequently acquired by MSSA and/or lost by MRSA.

Identification of an endemic MSSA clone

Due to its clinical relevance, much more attention has been given to MRSA than to MSSA. Although MSSA have been also frequently associated with acute infections, not only in the community but also in hospitals, their epidemiology is largely unexplored. We have contributed in Chapter III to a deeper knowledge of the MSSA population structure, and their geographic distribution and dynamics over almost two decades in Portugal.

MSSA isolates mainly from infection but also colonization, with both hospital and community origin, collected for over 19 years (1992–2011), covering North, Center and South of Portugal were analyzed. Taking all data into consideration, we observed that the MSSA population is genetically very diverse, with no particular geographical distribution. Interestingly, although the MSSA population was widely heterogeneous, only a limited number (ten) of epidemic clonal types were present for almost two decades, including ST30-t012, ST5-t002, ST8-t008, ST15-t084, ST34-t166,

ST72-t148, ST1-t127, ST7-t091, ST398-t571 and ST34-t136. The genetic diversity found among MSSA population in Portugal, was already described as a characteristic feature associated with MSSA (21, 24). Moreover, the MSSA clonal types we found in Portugal have been already described in other European countries (4, 25, 45, 52) and in the United States (42), although in different proportions. However, a different MSSA population structure was observed in Asian and African countries. In Taiwan ST508 (SLVST45)-t015, ST25-t340, ST121-t645, ST59-t437 and ST188-t189 (9) were the most predominant MSSA clones. In Cameroon, Madagascar, Morocco, Niger and Senegal, 58% of MSSA isolates from infection belonged to clonal types ST121-t314, ST15-t084, ST1851(SLV of ST1)-t127 and ST152-t355 (6). However the explanation for this variation in MSSA population structure between the different countries are poorly explored and understood.

We observed that the ST30-t012 was the most epidemic clone, being present in the entire period and all over the country, whereas the other nine clones were intermittently detected over time. The clone (ST30-t012) was detected for the first time in the 1950s, initially reported in the hospitals, in Australia, Great Britain, Canada, and the USA, in the form of the penicillin-resistant phage-type 80/81 clone (MSSA) [revised in (12)]. Finally, more recently, it has emerged also as the contemporary CA-MRSA epidemic Southwest Pacific clone (SWP) (ST30-IV, t012) (12). The evolution of ST30 (and CC30) has been extensively studied (12, 41). Collectively, the authors found that the phage-type 80/81 and SWP clone evolved towards an enhanced virulence capacity, when compared to the vast majority of healthcare-associated CC30, in particular the EMRSA-16 (ST36-II), a clone exclusively associated to the hospitals, where non-synonymous single nucleotide polymorphisms (SNPs) occurred in the alpha-hemolysin (*hla*), accessory gene regulator C (*agrC*) and *crtM* (gene encoding squalene desaturase), resulting in non-functional genes that impact on virulence capacity of this clone. Accordingly, the most epidemic MSSA clonal type found in Portugal may be also a descendent of phage type 80/81, where particular genetic features may be responsible for its higher capacity of colonization and invasion. In this context, it would be interesting to identify putative genetic factors responsible for the wide dissemination ability of these clones through the construction of a transposon mutant library for factors involved in adhesion, virulence and invasion.

Interestingly, the three epidemic MSSA clonal types ST8-t008, ST72-t148 and ST1-t127 are genetically related with the worldwide CA-MRSA epidemic clones were isolated in Portugal nearly the beginning of the CA-MRSA era, in 1996. We may speculate that these MSSA resulted from SCC*mec* loss from correspondent CA-MRSA backgrounds previously introduced in Portugal (ST8-IV, ST72-IV, ST1-IV) (1, 10, 16, 54, 59). Although, introduction of these MSSA from abroad in 1996 could not be excluded, since the first reports of strains belonging to these CA-MRSA epidemic clones in Portugal were only registered after 2006 (1). Comparative whole genome sequencing of MSSA and MRSA strains belonging to ST8, ST72 and ST1 could help to understand how related they are in fact.

Other less minor clonal types ST121, ST15 and ST25 persist in the Portuguese community for as long as 20 years, showing a considerable epidemicity. The first descriptions of these clones date back to 1950s in Denmark (22). But nowadays, these clones have a worldwide geographical distribution, particularly in Europe (10, 24, 51, 64). In Portugal, ST121 was first reported as MRSA, in a hospital survey between 2003-2006 (ST121-IV) (2), and only more recently as MSSA carrying PVL and causing SSTIs (19 out of the 35 MSSA) in children attending a pediatric emergency department of a central hospital in Lisbon (10).

MRSA relatedness with MSSA

MRSA emerged a limited number of times by the introduction of SCC*mec* in a few MSSA epidemic lineages. Therefore, the evaluation of MSSA population and the genetic relatedness with MRSA is essential to understand possible origins and dynamics of the MRSA clones. One of the aspects we wanted to clarify was whether the CA-MRSA had emerged from an established MSSA population or otherwise has been imported from other countries. For this purpose, the MSSA population found in the community, but also in hospitals, was compared with the MRSA included in our studies.

Altogether, data showed that the MSSA with genetic backgrounds similar to the emergent CA-MRSA epidemic clones were found nowadays in Portugal (See Figure 1), suggesting that CA-MRSA could have emerged locally from this established MSSA population, although introduction from abroad resultant from immigration/emigration fluxes from different countries cannot be excluded. Of note, the presence of the European clone in Portugal might result from introduction from abroad, since no MSSA counterpart was found circulating in the community and was ever

described in the past in Portugal. Its origin could have been the African countries, wherein MSSA strains belonging to ST80 were described to exist (20).

Alternatively, the hypothesis of SCC*mec* loss from MRSA should be also equated, not only from CA-MRSA clones, but also from HA-MRSA clones such as EMRSA-15 and NY/JP found in the community, in which SCC*mec* carriage may constitute a fitness cost. The case of ERMSA-15 is illustrative of this hypothesis. This clone was presumptively introduced in Portugal almost a decade ago (2), with a wide distribution in many Portuguese hospitals (1, 16, 19). However, recently we are observing simultaneously, the emergence of EMRSA-15 and ST22-MSSA in the community. These observations suggest that when EMRSA-15 was displaced to the community, might have lost SCC*mec* probably due to lack (high) of antibiotic pressure. However, this is debatable since some authors reported that antimicrobials reduction probably is not sufficient to encourage the loss of SCC*mec* from EMRSA-15 (ST22-IV), where the carriage of the respective cassette may not constituted a fitness disadvantage (31).

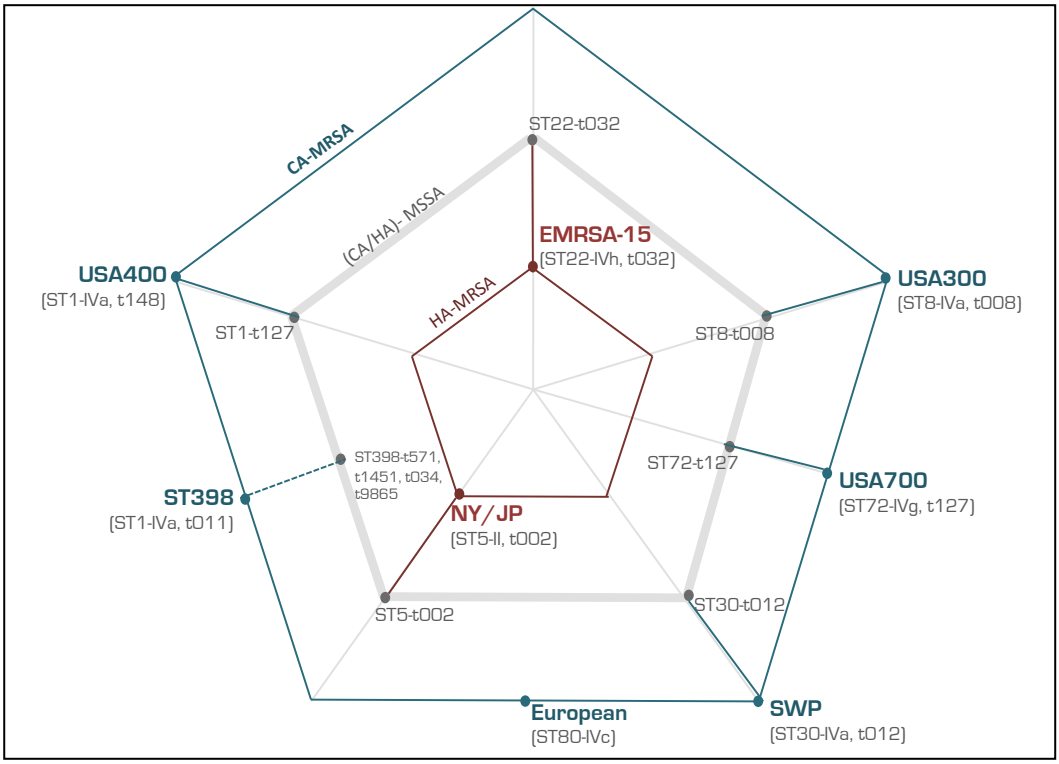


Figure 1. Genetic relatedness between MRSA and MSSA epidemic clones found in the community in Portugal (2009/2011) (dashed line - indicates not so close relatedness, MSSA and MRSA ST398 clone with different *spa* types).

In this context, we believe that MSSA should be included in future epidemiological surveys, not only because they were already identified as potential pathogens in Portugal, but also because they can give rise to MRSA, a major worldwide public health concern. Given the pattern of MSSA variation observed in our study (19 years period), we believe that the screening of out(in)patients, with intervals of 5 years, should be sufficient to evaluate the MSSA population structure and temporal dynamics in order to control possible emergent MSSA reservoirs.

Insights into alpha-hemolysin: heterogeneous expression profiles

Several studies emphasized the importance of alpha-hemolysin (Hla) as the major virulence determinant implicated in *S. aureus* pathogenesis, but the breadth of genetic diversity, evolution and its importance in virulence of CA-MRSA was only poorly explored. Although CA-MRSA were described to express more *hla* than HA-MRSA, the impact of *hla* was never assessed in a large and representative *S. aureus* collection, and has been restricted almost exclusively to the USA300 clone (7).

In order to answer to these questions, in Chapter IV we evaluated *hla* nucleotide sequence variation and gene expression among the main epidemic and minor (HA)CA-MRSA/MSSA clones. We described for the first time evidences that the Hla evolved together with the genetic background. This was previously shown to occur for other virulence genes in *S. aureus* such as adhesins (*clfA*, *clfB*, *fnbA*, *map*, *sdrC*, *spa*, *ebpS*, *fnbB*, *sdrD*, and *sdrE*) (34), suggesting that the virulence genes are very important for *S. aureus* survival and evolution. However, the promoter gene had a dissimilar evolutionary pathway, probably in response to different environmental stresses that demanded for different levels of gene expression.

The data obtained confirmed the general assumption that CA-MRSA are more virulent than HA-MRSA clones (5, 8, 37, 46), since CA backgrounds showed a higher levels of *hla* expression than the HA backgrounds. Moreover, no significant differences in expression rates were oberved between MRSA and MSSA. The most striking finding regards to the fact that the *hla* expression within the (HA)CA-MRSA clones were not uniform, but rather heterogeneous – a high strain-to-strain variation in the level of gene expression was detected in highly related isolates. On the other hand, we observed that two isolates, representative of the two most common clones found in the hospitals in Portugal (EMRSA-15 and NY/JP), had a high *hla* expression level. Interestingly, these

are the two main clones also present in the community in Portugal. We may speculate that the increased expression of *hla* can result from an adaptation of these two clones to the community environment.

The results obtained alerted us to the fact that in what concerns to virulence potential in *S. aureus*, conclusions taken for one or very few strains cannot be generalized to the entire population, or even for a group of related strains. We believe that this major conclusion can be extrapolated to all remaining *S. aureus* virulence factors. However, it remains unanswered to what extent this heterogeneous expression profiles impact on phenotypic activity, protein production and virulence. This could be achieved doing additional assays, namely by performing hemolysis assays immunoblotting, and by using *in vivo* animal infection models.

Model for the origin of MRSA in the community in Portugal

Considering the data obtained in this Thesis (Chapter II and III), together with data available from other national HA-MRSA and MSSA surveillance studies, we draw the model depicted in Figure 2. In this model we illustrate the main MRSA and MSSA clones in hospitals and community in Portugal. Moreover, we speculated on the possible origins, and dynamics, of the MRSA clones present nowadays in the community.

The model proposed considers that the MRSA present nowadays in the community result from: i. dissemination of nosocomial MRSA, ii) SCC*mec* acquisition by the established MSSA and iii) MRSA introduction from abroad.

Based on the successive waves of clonal replacement reported in Portuguese hospitals along 16 years (1) in combination with actual HA-MRSA clonal predominance (17, 19), we may consider two periods (Figure 2): (i). first period (1990-2001) - where the clonal replacement process occurred in a relatively short time and where the emergent clone completely replaced the precedent one; in this period the prevalent clones were the early multidrug resistant clones, carrying the largest SCC*mec* cassettes (SCC*mec* I and III); (ii) second period (2001 to 2011) - a major change in the HA-MRSA epidemiology occurred with the emergence of the EMRSA-15 clone in 2001, followed by the emergence of the NY/JP clone in 2006, however, this time, clonal replacement did not occurred and rather co-existence of these two clones is observed until our days; these two clones carry smaller

SCC*mec* cassettes, type IV and II, and particularly type IV characteristically do not accumulate multidrug resistance.

If in the former period, the influence of the dominant nosocomial clones in the MRSA community dynamics was not observed, since Portuguese (ST239-IIIvar), Iberian (ST247-IIA) and Brazilian (ST238-III) clones were not fit to survive in the community, the same cannot be assumed after 2001 with the EMRSA-15 clone, and also NY/JP, since these clones have already demonstrated to have the ability to survive and disseminate in the community (27, 39). Our data and model are in accordance with this, since a great proportion of MRSA present in the community belonged to the EMRSA-15 clone (and NY/JP also), clearly indicating the blurring of the boundaries between the hospital and the community in Portugal.

In fact, public buses were already described as main reservoirs and vehicle of dissemination of the EMRSA-15 and also NY/JP clones between these two environments, particularly the buses circulating close to hospitals (11, 56). Contaminated hands of passengers (11) may also work as possible vehicle of dissemination of the HA-MRSA clones in the Portuguese community. In addition, if the boundaries in fact are blurred, then the introduction of CA-MRSA isolates into the hospital might also be occurring, a hypothesis not far from the reality since USA300 related background was already described in the hospital setting in Portugal (1, 16). This entry may in the future lead to the accumulation of antimicrobial resistance in already highly virulent clones, resulting in the local emergence of superbug that convey, simultaneously, multiple antimicrobial resistance and virulence, which is already a reality in the USA (61)

The other two hypotheses proposed for MRSA origin in the community (ii and iii), particularly addresses the CA-MRSA clones. The introduction of CA-MRSA from abroad is a possibility, since in our studies we observed the existence of USA700 and USA300 prototype clones with multidrug resistance profiles, carrying ACME and PVL, that could have been originated from Asia and USA respectively, and spread into the Portuguese community. However the entry of these clones into the hospitals, followed by escaping into the community after accumulation of antimicrobial resistance cannot be excluded. On the other hand, acquisition of SCC*mec* by the established MSSA population or the introduction of MSSA genetic background related with CA-MRSA from abroad followed by SCC*mec* acquisition should be also considered.

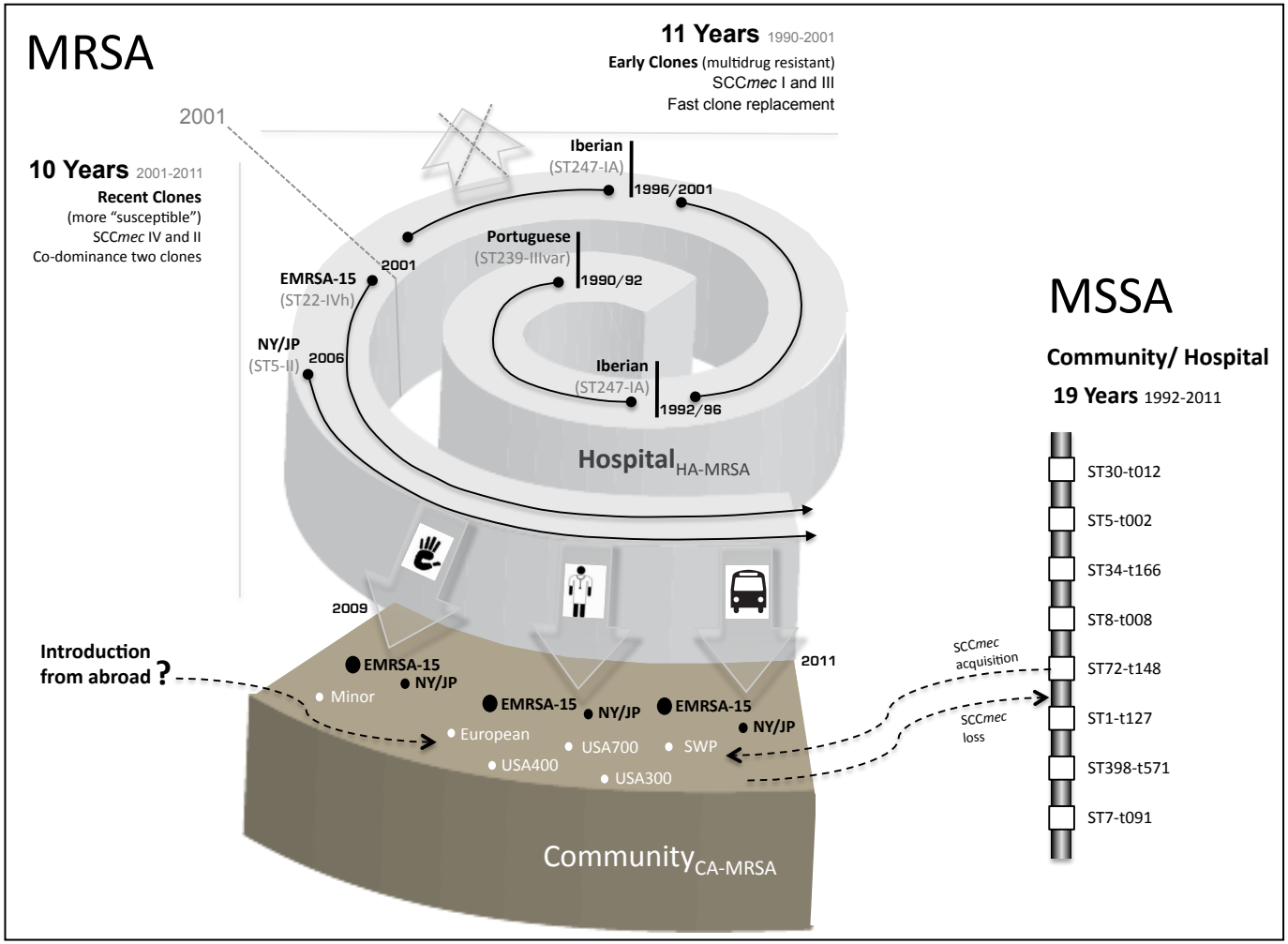


Figure 2. Model for the origin of MRSA in the community in Portugal. MRSA population structure in the hospitals (1990-2011) and in the community (our studies) (2009/2011).

REFERENCES

1. **Aires-de-Sousa, M., B. Correia, and H. de Lencastre.** 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:**2912-7.
2. **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.
3. **Amorim, M. L., C. Vasconcelos, D. C. Oliveira, A. Azevedo, E. Calado, N. A. Faria, M. Pereira, A. P. Castro, A. Moreira, E. Aires, J. M. Cabeda, M. H. Ramos, J. M. Amorim, and H. de Lencastre.** 2009. Epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) nasal colonization among patients and healthcare workers in a Portuguese hospital: a pre-intervention study toward the control of MRSA. Microb Drug Resist **15:**19-26.
4. **Argudin, M. A., V. Argumosa, M. C. Mendoza, B. Guerra, and M. R. Rodicio.** 2013. Population structure and exotoxin gene content of methicillin-susceptible *Staphylococcus aureus* from Spanish healthy carriers. Microb Pathog **54:**26-33.
5. **Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. 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.
6. **Breurec, S., C. Fall, R. Pouillot, P. Boisier, S. Brisse, F. Diene-Sarr, S. Djibo, J. Etienne, M. C. Fonkoua, J. D. Perrier-Gros-Claude, C. E. Ramarokoto, F. Randrianirina, J. M. Thiberge, S. B. Zriouil, B. Garin, and F. Laurent.** 2011. Epidemiology of methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of Panton-Valentine leukocidin genes. Clin Microbiol Infect **17:**633-9.
7. **Bubeck Wardenburg, J., T. Bae, M. Otto, F. R. Deleo, and O. Schneewind.** 2007. Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. Nat Med **13:**1405-6.
8. **Burlak, C., C. H. Hammer, M. A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo.** 2007. Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. Cell Microbiol **9:**1172-90.
9. **Chen, F. J., L. K. Siu, J. C. Lin, C. H. Wang, and P. L. Lu.** 2012. Molecular typing and characterization of nasal carriage and community-onset infection methicillin-susceptible *Staphylococcus aureus* isolates in two Taiwan medical centers. BMC Infect Dis **12:**343.
10. **Conceicao, T., M. Aires-de-Sousa, N. Pona, M. J. Brito, C. Barradas, R. Coelho, T. Sardinha, L. Sancho, G. de Sousa, C. Machado Mdo, and H. de Lencastre.** 2011. High prevalence of ST121 in community-associated methicillin-susceptible *Staphylococcus aureus* lineages responsible for skin and soft tissue infections in Portuguese children. Eur J Clin Microbiol Infect Dis **30:**293-7.
11. **Conceicao, 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.
12. **DeLeo, F. R., A. D. Kennedy, L. Chen, J. Bubeck Wardenburg, S. D. Kobayashi, B. Mathema, K. R. Braughton, A. R. Whitney, A. E. Villaruz, C. A. Martens, S. F. Porcella, M. J. McGavin, M. Otto, J. M. Musser, and B. N. Kreiswirth.** 2011. Molecular differentiation of historic phage-type 80/81 and contemporary epidemic *Staphylococcus aureus*. Proc Natl Acad Sci U S A **108:**18091-6.
13. **DGS.** 2012. Direção-Geral da Saúde, Departamento da Qualidade na Saúde. Prevalência de Infeção Adquirida no Hospital e do Uso de Antimicrobianos nos Hospitais Portugueses, Inquérito 2012. Portugal:DGS. www.dgs.pt [On line].
14. **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.

15. **ECDC.** 2010. European Centre for Disease Prevention and Control. Surveillance of antimicrobial consumption in Europe, 2010. Stockholm, March 2013. http://www.ecdc.europa.eu/ [Online].
16. **Espadinha, D., N. A. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, and H. de Lencastre.** 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.
17. **Espadinha, D., N. A. Faria, M. Miragaia, L. M. Lito, J. Melo-Cristino, H. de Lencastre, and N. Medicos Sentinela.** 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.
18. **Fang, H., G. Hedin, G. Li, and C. E. Nord.** 2008. Genetic diversity of community-associated methicillin-resistant *Staphylococcus aureus* in southern Stockholm, 2000-2005. Clin Microbiol Infect **14:**370-6.
19. **Faria, N. A., M. Miragaia, H. de Lencastre, and C. Multi Laboratory Project.** 2013. Massive dissemination of methicillin resistant *Staphylococcus aureus* in bloodstream infections in a high MRSA prevalence country: establishment and diversification of EMRSA-15. Microb Drug Resist **19:**483-90.
20. **Ghebremedhin, B., M. O. Olugbosi, A. M. Raji, F. Layer, R. A. Bakare, B. Konig, and W. Konig.** 2009. Emergence of a community-associated methicillin-resistant *Staphylococcus aureus* strain with a unique resistance profile in Southwest Nigeria. J Clin Microbiol **47:**2975-80.
21. **Goering, R. V., R. M. Shawar, N. E. Scangarella, F. P. O'Hara, H. Amrine-Madsen, J. M. West, M. Dalessandro, J. A. Becker, S. L. Walsh, L. A. Miller, S. F. van Horn, E. S. Thomas, and M. E. Twynholm.** 2008. Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials. J Clin Microbiol **46:**2842-7.
22. **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.
23. **Gonzalez-Dominguez, M., C. Seral, Y. Saenz, S. Salvo, M. J. Gude, N. Porres-Osante, C. Torres, and F. J. Castillo.** 2012. Epidemiological features, resistance genes, and clones among community-onset methicillin-resistant *Staphylococcus aureus* (CO-MRSA) isolates detected in northern Spain. Int J Med Microbiol **302:**320-6.
24. **Grundmann, H., D. M. Aanensen, C. C. van den Wijngaard, B. G. Spratt, D. Harmsen, and A. W. Friedrich.** 2010. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med **7:**e1000215.
25. **Hallin, M., O. Denis, A. Deplano, R. De Mendonca, R. De Ryck, S. Rottiers, and M. J. Struelens.** 2007. Genetic relatedness between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*: results of a national survey. J Antimicrob Chemother **59:**465-72.
26. **Holden, M. T., L. Y. Hsu, K. Kurt, L. A. Weinert, A. E. Mather, S. R. Harris, B. Strommenger, F. Layer, W. Witte, H. de Lencastre, R. Skov, H. Westh, H. Zemlickova, G. Coombs, A. M. Kearns, R. L. Hill, J. Edgeworth, I. Gould, V. Gant, J. Cooke, G. F. Edwards, P. R. McAdam, K. E. Templeton, A. McCann, Z. Zhou, S. Castillo-Ramirez, E. J. Feil, L. O. Hudson, M. C. Enright, F. Balloux, D. M. Aanensen, B. G. Spratt, J. R. Fitzgerald, J. Parkhill, M. Achtman, S. D. Bentley, and U. Nubel.** 2013. A genomic portrait of the emergence, evolution and global spread of a methicillin resistant *Staphylococcus aureus* pandemic. Genome Res.
27. **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.
28. **Horvath, A., O. Dobay, S. Kardos, A. Ghidan, A. Toth, J. Paszti, E. Ungvari, P. Horvath, K. Nagy, S. Zissman, and M. Fuzi.** 2012. Varying fitness cost associated with resistance to

fluoroquinolones governs clonal dynamic of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis **31:**2029-36.

29. **Jappe, U., D. Heuck, B. Strommenger, C. Wendt, G. Werner, D. Altmann, and W. Witte.** 2008. *Staphylococcus aureus* in dermatology outpatients with special emphasis on community-associated methicillin-resistant strains. J Invest Dermatol **128:**2655-64.
30. **Joshi, G. S., J. S. Spontak, D. G. Klapper, and A. R. Richardson.** 2011. Arginine catabolic mobile element encoded *speG* abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. Mol Microbiol **82:**9-20.
31. **Knight, G. M., E. L. Budd, and J. A. Lindsay.** 2013. Large mobile genetic elements carrying resistance genes that do not confer a fitness burden in healthcare-associated meticillin-resistant *Staphylococcus aureus*. Microbiology **159:**1661-72.
32. **Knight, G. M., E. L. Budd, L. Whitney, A. Thornley, H. Al-Ghusein, T. Planche, and J. A. Lindsay.** 2012. Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time. J Antimicrob Chemother **67:**2514-22.
33. **Kramer, A., H. Wagenvoort, C. Ahren, I. Daniels-Haardt, P. Hartemann, H. Kobayashi, A. Kurcz, J. Picazo, G. Privitera, and O. Assadian.** 2010. Epidemiology of MRSA and current strategies in Europe and Japan. GMS Krankenhhyg Interdiszip **5:**Doc01.
34. **Kuhn, G., P. Francioli, and D. S. Blanc.** 2006. Evidence for clonal evolution among highly polymorphic genes in methicillin-resistant *Staphylococcus aureus*. J Bacteriol **188:**169-78.
35. **Lafaurie, M., R. Porcher, J. L. Donay, S. Touratier, and J. M. Molina.** 2012. Reduction of fluoroquinolone use is associated with a decrease in methicillin-resistant *Staphylococcus aureus* and fluoroquinolone-resistant *Pseudomonas aeruginosa* isolation rates: a 10 year study. J Antimicrob Chemother **67:**1010-5.
36. **Larsen, A. R., M. Stegger, S. Bocher, M. Sorum, D. L. Monnet, and R. L. Skov.** 2009. Emergence and characterization of community-associated methicillin-resistant *Staphyloccocus aureus* infections in Denmark, 1999 to 2006. J Clin Microbiol **47:**73-8.
37. **Li, M., G. Y. Cheung, J. Hu, D. Wang, H. S. Joo, F. R. Deleo, and M. Otto.** 2010. Comparative analysis of virulence and toxin expression of global community-associated methicillin-resistant *Staphylococcus aureus* strains. J Infect Dis **202:**1866-76.
38. **Manzur, A., A. M. Dominguez, M. Pujol, M. P. Gonzalez, E. Limon, A. Hornero, R. Martin, F. Gudiol, and J. Ariza.** 2008. Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. Clin Microbiol Infect **14:**377-80.
39. **Marchese, A., L. Gualco, E. Maioli, and E. Debbia.** 2009. Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int J Antimicrob Agents **34:**424-8.
40. **Maugat, S., A. de Rougemont, H. Aubry-Damon, M. E. Reverdy, S. Georges, F. Vandenesch, J. Etienne, and B. Coignard.** 2009. Methicillin-resistant *Staphylococcus aureus* among a network of French private-sector community-based-medical laboratories. Med Mal Infect **39:**311-8.
41. **McAdam, P. R., K. E. Templeton, G. F. Edwards, M. T. Holden, E. J. Feil, D. M. Aanensen, H. J. Bargawi, B. G. Spratt, S. D. Bentley, J. Parkhill, M. C. Enright, A. Holmes, E. K. Girvan, P. A. Godfrey, M. Feldgarden, A. M. Kearns, A. Rambaut, D. A. Robinson, and J. R. Fitzgerald.** 2012. Molecular tracing of the emergence, adaptation, and transmission of hospital-associated methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A **109:**9107-12.
42. **Miko, B. A., C. A. Hafer, C. J. Lee, S. B. Sullivan, M. A. Hackel, B. M. Johnson, S. Whittier, P. Della-Latta, A. C. Uhlemann, and F. D. Lowy.** 2013. Molecular characterization of methicillin-susceptible *Staphylococcus aureus* clinical isolates in the United States, 2004 to 2010. J Clin Microbiol **51:**874-9.
43. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, and D. A. Talan.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med **355:**666-74.

44. **Nazareth, R., J. Goncalves-Pereira, A. Tavares, M. Miragaia, H. de Lencastre, J. Silvestre, P. Freitas, E. Goncalves, F. Martins, V. Mendes, C. Tapadinhas, and P. Povoa.** 2012. Community-associated methicillin-resistant *Staphylococcus aureus* infection in Portugal. Rev Port Pneumol **18:**34-8.
45. **Nulens, E., E. E. Stobberingh, H. van Dessel, S. Sebastian, F. H. van Tiel, P. S. Beisser, and R. H. Deurenberg.** 2008. Molecular characterization of *Staphylococcus aureus* bloodstream isolates collected in a Dutch University Hospital between 1999 and 2006. J Clin Microbiol **46:**2438-41.
46. **Otto, M.** 2013. Community-associated MRSA: What makes them special? Int J Med Microbiol.
47. **Parienti, J. J., V. Cattoir, P. Thibon, G. Lebouvier, R. Verdon, C. Daubin, D. du Cheyron, R. Leclercq, and P. Charbonneau.** 2011. Hospital-wide modification of fluoroquinolone policy and meticillin-resistant *Staphylococcus aureus* rates: a 10-year interrupted time-series analysis. J Hosp Infect **78:**118-22.
48. **Planet, P. J., S. J. LaRussa, A. Dana, H. Smith, A. Xu, C. Ryan, A. C. Uhlemann, S. Boundy, J. Goldberg, A. Narechania, R. Kulkarni, A. J. Ratner, J. A. Geoghegan, S. O. Kolokotronis, and A. Prince.** 2013. Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine catabolic mobile element and *speG*-mediated adaptations for survival on skin. MBio **4:**e00889-13.
49. **Pomba, C., H. Hasman, L. M. Cavaco, J. D. da Fonseca, and F. M. Aarestrup.** 2009. First description of meticillin-resistant *Staphylococcus aureus* (MRSA) CC30 and CC398 from swine in Portugal. Int J Antimicrob Agents **34:**193-4.
50. **Ramalhinho, I., M. Ribeirinho, I. Vieira, and J. Cabrita.** 2012. [Evolution of outpatient antibiotic use in Portugal mainland 2000-2009]. Acta Med Port **25:**20-8.
51. **Rasigade, J. P., F. Laurent, G. Lina, H. Meugnier, M. Bes, F. Vandenesch, J. Etienne, and A. Tristan.** 2010. Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981-2007. J Infect Dis **201:**1589-97.
52. **Rijnders, M. I., R. H. Deurenberg, M. L. Boumans, J. A. Hoogkamp-Korstanje, P. S. Beisser, G. Antibiotic Resistance Surveillance, and E. E. Stobberingh.** 2009. Population structure of *Staphylococcus aureus* strains isolated from intensive care unit patients in the netherlands over an 11-year period (1996 to 2006). J Clin Microbiol **47:**4090-5.
53. **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.
54. **Sa-Leao, R., I. S. Sanches, I. Couto, C. R. Alves, and H. de Lencastre.** 2001. Low prevalence of methicillin-resistant strains among *Staphylococcus aureus* colonizing young and healthy members of the community in Portugal. Microb Drug Resist **7:**237-45.
55. **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 ST22-MRSA-IV. Antimicrob Agents Chemother **55:**1896-905.
56. **Simoes, R. R., M. Aires-de-Sousa, T. Conceicao, 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.
57. **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, P. H. Van, and A. S. 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.

58. **Sonnevend, A., I. Blair, M. Alkaabi, P. Jumaa, M. Al Haj, A. Ghazawi, N. Akawi, F. S. Jouhar, M. B. Hamadeh, and T. Pal.** 2012. Change in meticillin-resistant *Staphylococcus aureus* clones at a tertiary care hospital in the United Arab Emirates over a 5-year period. J Clin Pathol **65:**178-82.

59. **Tavares, D. A., R. Sa-Leao, M. Miragaia, and H. de Lencastre.** 2010. Large screening of CA-MRSA among *Staphylococcus aureus* colonizing healthy young children living in two areas (urban and rural) of Portugal. BMC Infect Dis **10:**110.

60. **Tenover, F. C., and R. V. Goering.** 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. J Antimicrob Chemother **64:**441-6.

61. **Tenover, F. C., I. A. Tickler, R. V. Goering, B. N. Kreiswirth, J. R. Mediavilla, D. H. Persing, and M. Consortium.** 2012. Characterization of nasal and blood culture isolates of methicillin-resistant *Staphylococcus aureus* from patients in United States Hospitals. Antimicrob Agents Chemother **56:**1324-30.

62. **Thibaut, S., J. Caillon, C. Huart, G. Grandjean, P. Lombrail, G. Potel, F. Ballereau, and R. Microbiology laboratories of the Pays de la Loire.** 2010. Susceptibility to the main antibiotics of *Escherichia coli* and *Staphylococcus aureus* strains identified in community acquired infections in France (MedQual, 2004-2007). Med Mal Infect **40:**74-80.

63. **van Trijp, M. J., D. C. Melles, W. D. Hendriks, G. A. Parlevliet, M. Gommans, and A. Ott.** 2007. Successful control of widespread methicillin-resistant *Staphylococcus aureus* colonization and infection in a large teaching hospital in the Netherlands. Infect Control Hosp Epidemiol **28:**970-5.

64. **Vorobieva, V., T. Bazhukova, A. M. Hanssen, D. A. Caugant, N. Semenova, B. C. Haldorsen, G. S. Simonsen, and A. Sundsfjord.** 2008. Clinical isolates of *Staphylococcus aureus* from the Arkhangelsk region, Russia: antimicrobial susceptibility, molecular epidemiology, and distribution of Panton-Valentine leukocidin genes. APMIS **116:**877-87.

65. **Vourli, S., H. Vagiakou, G. Ganteris, M. Orfanidou, M. Polemis, A. Vatopoulos, and H. Malamou-Ladas.** 2009. High rates of community-acquired, Panton-Valentine leukocidin (PVL)-positive methicillin-resistant *S. aureus* (MRSA) infections in adult outpatients in Greece. Euro Surveill **14**.