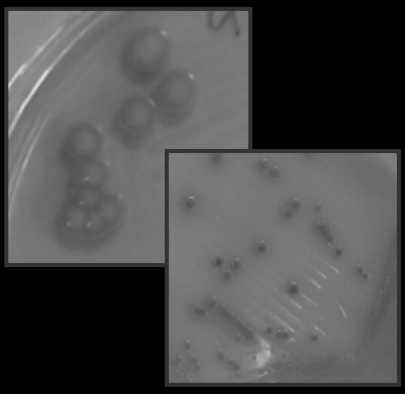
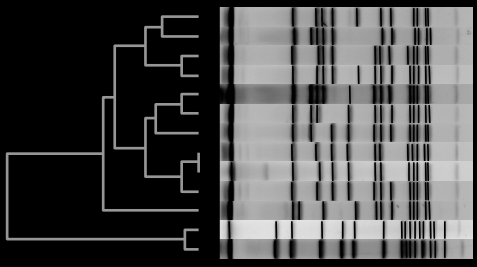
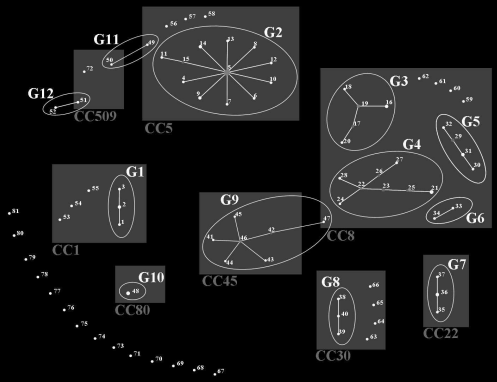
Staphylococcus aureus :

towards a comprehensive view on epidemiology and clonal spread

Teresa Conceição







Universidade Nova de Lisboa
Instituto de Tecnologia Química e Biológica
Oeiras, 2011

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ABSTRACT

Staphylococcus aureus is known for its extraordinary capacity of colonizing asymptomatically the anterior nares of humans. A high versatility allied to the successive accumulation of resistance to almost all clinically available antibiotics, made *S. aureus* a major human pathogen worldwide. Soon after the introduction of methicillin into clinical practice, methicillin resistant *S. aureus* (MRSA) emerged as a major pathogen in hospitals worldwide, and more recently in the community as well. Methicillin susceptible *S. aureus* (MSSA) are also an important problem. MSSA is responsible from mild to severe invasive infections, commonly due to enhanced virulence content, rather than accumulation of resistance determinates. However, many gaps in the knowledge of the epidemiology of MSSA remain to be clarified.

The aim of the work presented in this Thesis was to obtain new insights into different *S. aureus* populations, assessing the genetic background of MSSA and MRSA populations, from colonization and infection, recovered from both hospital and community settings using well established techniques and a new typing method.

The comparison of the genetic background of two MSSA collections (from Portuguese nosocomial and community settings and Cape Verde hospitals) with contemporary nosocomial MRSA populations, revealed new trends on the origins of the dominant MRSA lineages. Except for the MRSA Pediatric clone that might have originated in Portugal by the introduction of SCC*mec* type VI into the previously existing MSSA ST5 lineage, the non-overlapping of the remaining MSSA and MRSA genetic backgrounds suggested that the major MRSA clones identified in Portuguese hospitals were imported from abroad. Moreover, the fact that no MRSA coexisted with the MSSA population in Cape Verde further supports the evidence that the introduction of SCC*mec* into sensitive clones is most likely a relatively infrequent event that seems to depend not exclusively on the presence of a successful MSSA lineage.

In parallel to the burden of MRSA in the hospitals, CA-MRSA, first described in the early 1990's, became dispersed worldwide through few major pandemic lineages. In contrast to other countries, the CA-MRSA prevalence in Portugal, estimated by previous studies of nasal carriage, was very low. The screening of skin and soft tissue infections in a pediatric community, presented in this Thesis, confirmed these rates. Moreover, we reported the first isolate in Portugal belonging to ST80-IV, the major CA-MRSA lineage found in Europe and usually associated to the production of the Panton-Valentine leukocidine (PVL).

An unusual high toxin content was found in the Cape Verde MSSA collection, with 35% of the isolates producing PVL and 8% harboring the exfoliative toxin D gene, which seems an epidemic advantage in an environment with very low antimicrobial pressure, driven by the restricted use of antibiotics.

By contrast to the low prevalence of CA-MRSA, the MRSA prevalence in Portuguese hospitals is one of the highest in Europe, reaching 53% in 2008. Whereas the epidemiology of nosocomial MRSA had been very well characterized in continental Portugal, there was no information concerning the clonal nature of MRSA in the Portuguese islands, namely in the Azores archipelago. To fill this gap, a collection of MRSA recovered in an Azorean hospital was studied and showed the presence of the EMRSA-15 clone (ST22-IVh), followed by the Pediatric clone (ST5-VI), which is in part, in agreement with the continental Portugal scenario. Moreover, PVL was described for the first time in association with the Pediatric clone.

To understand the MRSA clonal evolution in Hungary, we performed a 10-year surveillance study, which revealed a clonal replacement during this period. The Hungarian clone (ST239-III), predominant in hospitals in 1994-1998, was gradually replaced by the Southern German clone (ST228-I) and the New York/ Japan epidemic clone (ST5-II), representing 85% of the 2001–2004 isolates. This study described, for the first time, the co-dominance and extensive spread of the New York/ Japan clone in a European country.

The definition of clonal lineages and the clarification of their evolution in the hospital and in the community, require the use of up-to-date molecular typing

methods such as pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST) and the determination of the SCC*mec* type in MRSA isolates, which are valuable tools for *S. aureus* characterization. In addition to these well-established methods used for the characterization of the collections studied in this Thesis, we validated a new typing method: the staphylococcal interspersed repeat analysis (SIRUs). It is a multilocus variable number of tandem repeats (VNTR) analysis (MLVA) based methodology that we applied to a temporal and geographical diverse *S. aureus* collection. The observed high discriminatory power of the SIRU method, along with its apparent concordance with MLST, makes it potentially valuable not only for *S. aureus* short-term epidemiological investigations, but for population dynamics as well.

RESUMO

Staphylococcus aureus é conhecido pela sua extraordinária capacidade de colonização assintomática das narinas no ser humano. A sua versatilidade aliada à sucessiva acumulação de resistência à grande maioria dos antibióticos disponíveis clinicamente, faz de *S. aureus* um dos principais agentes patogénicos em todo o mundo. Logo após a introdução da meticilina na prática clínica, *S. aureus* resistente à meticilina (MRSA, do inglês "methicillin-resistant *Staphylococcus aureus*") surgiu como um importante agente patogénico nos hospitais, e mais recentemente também na comunidade. No entanto, alterações na epidemiologia de *S. aureus* não estão restritas a MRSA, uma vez que *S. aureus* susceptíveis (MSSA, do inglês "methicillin-susceptible *Staphylococcus aureus*") são também um problema crescente. MSSA tornou-se responsável por uma grande variedade de infecções invasivas com maior ou menor gravidade, geralmente devido ao elevado potencial de virulência, e não à acumulação de determinantes de resistência. Contudo, muito do conhecimento da epidemiologia de MSSA continua por esclarecer.

Pretendeu-se com o trabalho apresentado nesta Tese obter novos dados sobre diferentes populações de *S. aureus* através da utilização de técnicas de tipagem bem estabelecidas e de um método de tipagem descrito recentemente. Procurámos determinar o perfil genético de populações de MRSA e MSSA, tanto de colonização como de infecção, e isoladas não só de ambiente hospitalar como também da comunidade.

A comparação entre a componente genética de duas colecções de MSSA, isoladas em hospitais e na comunidade Portuguesa e em hospitais de Cabo Verde, com as populações de MRSA hospitalares contemporâneas, revelou novas pistas sobre as origens das maiores linhagens de MRSA. Foi-nos possível demonstrar que os principais clones de MRSA identificados nos hospitais Portugueses foram provavelmente importados do estrangeiro. Esta conclusão baseou-se na não existência de sobreposição das características genéticas entre

os clones de MSSA e MRSA. A única excepção é o clone Pediátrico que poderá ter tido a sua origem em Portugal através da introdução do elemento móvel transportador do gene *mec* (SCC*mec*, do inglês "staphylococcal chromosomal cassette *mec*") tipo VI na linhagem de MSSA ST5 existente anteriormente, Por outro lado, a não existência de MRSA em Cabo Verde no mesmo período temporal em que se isolou a colecção de MSSA reforça a evidência de que a introdução do SCC*mec* em clones sensíveis é provavelmente um acontecimento pouco frequente que parece não depender apenas da presença de uma linhagem de MSSA bem sucedida.

No início dos anos 90 começaram a ser descritas linhagens de MRSA isoladas na comunidade (CA-MRSA, do inglês "community-acquired methicillin-resistant *Staphylococcus aureus"*), e que actualmente se encontram dispersas por todo o mundo. Em contraste com outros países, a prevalência de CA-MRSA em Portugal, estimada por estudos anteriores de colonização nasal, parece ser ainda reduzida. O rastreio de infecções da pele e tecidos moles numa comunidade pediátrica, apresentado nesta Tese, confirmou estas taxas. Além disso, descrevemos o primeiro isolado ST80-IV em Portugal, pertencente a uma das principais linhagens de CA-MRSA encontradas na Europa e, geralmente, associada à produção da leucocidina de Panton-Valentine (PVL do inglês "Panton-Valentine leukocidine").

Na colecção de MSSA de Cabo Verde foi encontrado um elevado número de isolados produtores de toxinas, nomeadamente PVL (35%) e toxina esfoliativa D (8%). A produção de toxinas por estirpes sensíveis parece ser uma vantagem num ambiente onde o uso restrito de antibióticos conduz a uma reduzida pressão antimicrobiana.

Em contraste com a baixa prevalência de CA-MRSA, a prevalência de MRSA nos hospitais Portugueses é uma das mais elevadas da Europa, tendo atingido os 53% em 2008. Apesar da epidemiologia de MRSA em ambiente hospitalar estar bem caracterizada em Portugal continental, até à data não existia

qualquer informação sobre a natureza clonal dos MRSA nas ilhas Portuguesas, nomeadamente no arquipélago dos Açores. Para preencher esta lacuna, estudou-se uma colecção de MRSA de um hospital Açoriano tendo-se observado uma predominância do clone EMRSA-15 (ST22-IV), seguida do clone Pediátrico (ST5-VI). Este cenário é, em parte, concordante com o de Portugal continental. Além disso, neste estudo, a toxina PVL foi descrita pela primeira vez em associação com o clone Pediátrico.

Para compreender a evolução clonal dos MRSA nos hospitais na Hungria, foi realizado um estudo de vigilância ao longo de 10 anos, que revelou um processo de substituição clonal. O clone Húngaro (ST239-III), predominante entre 1994-1998, foi gradualmente substituído pelos clones *Southern German* (ST228-I) e *New York/ Japan* (ST5-II), que representam 85% dos isolados no período de 2001-2004. Este estudo descreve, pela primeira vez, a co-dominância e disseminação global do clone *New York/ Japan* num país Europeu.

A definição de linhagens clonais no hospital e na comunidade e a determinação da sua história evolutiva, requerem a utilização de métodos de tipagem molecular actualizados, que são ferramentas valiosas na caracterização de *S. aureus*. Os métodos principais no estudo de *S. aureus* são: a electroforese em gel de campo pulsado (PFGE do inglês "pulsed-field gel electrophoresis"), a sequenciação do gene que codifica a proteína A ("*spa* typing"), a sequenciação de regiões internas de sete genes essenciais ao metabolismo bacteriano (MLST, do inglês "multi-locus sequence typing"), ou a determinação do tipo estrutural do SCC*mec* em isolados de MRSA. Além destes métodos bem estabelecidos e utilizados na caracterização das colecções estudadas nesta Tese, procedemos também à validação de um novo método de tipagem: a análise de regiões repetitivas intercaladas no genoma de *Staphylococcus* (SIRU do inglês "staphylococcal interspersed repeat units"). Esta metodologia é baseada na análise do número variável de repetições adjacentes em diferentes *locus* no genoma (MLVA do inglês "multilocus variable number of tandem repeats (VNTR) analysis"), que foi aplicada a uma colecção de *S. aureus* muito diversa tanto

temporal como geograficamente. O elevado poder discriminatório do método SIRU, juntamente com a sua aparente concordância com os resultados de MLST, torna o método potencialmente valioso, não só para estudos epidemiológicos a curto prazo, mas também para estudos de dinâmica populacional de *S. aureus*.

Thesis Outline

The present Thesis describes the genetic backgrounds of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* populations involved in colonization and infection, using a combination of well established molecular typing methods as well as the validation of a new typing strategy.

Relevant aspects of the biology of *S. aureus* and an overview of the most used typing methods for strain characterization are presented in Chapter I, General introduction. Insights into the actual molecular epidemiology of *S. aureus* as well as the role of selected virulence factors in pathogenicity are also reviewed.

The subsequent chapters have been assembled into three main sections:

Methicillin-susceptible *S. aureus* (MSSA): a colonizer but a clinical threat

In Chapter II, the genetic background of a collection of MSSA isolates recovered from both hospital and community settings in Portugal, is characterized and compared with the dominant MRSA clones circulating, during the same period, in the same geographical areas.

S. aureus isolates from skin and soft tissue infections in children attending a pediatric emergency unit in a Portuguese hospital are presented in Chapter III. The high virulence gene content and the clonal structure of the bacterial isolates are compared with the European scenario.

Chapter IV describes a collection of MSSA colonization isolates recovered in two African hospitals in Cape Verde islands showing a high prevalence of the Panton-Valentine leukocidin virulence determinant.

Methicillin-resistant *S. aureus* (MRSA): the still most frequent resistant pathogen in the nosocomial setting

Chapters V and VI describe the molecular characterization of nosocomial MRSA collections.

Chapter V describes the current MRSA population structure in a Portuguese central hospital located in a geographically restricted insular territory (the Azores islands).
In Chapter VI, two temporally separated (1994-1998 and 2001-2004) collections of MRSA from different Hungarian hospitals allowed the establishment of the temporal evolution of the epidemic clones in Central Europe.

Typing methods: a constant need of improvement

In Chapter VII, a new multilocus variable tandem repeated based method is validated against a temporally and geographically diverse collection of *S. aureus*, previously characterized by well established molecular typing methods.

The main achievements of this work are summarized and discussed in Chapter VIII.

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Chapter I

General Introduction

1. *Staphylococcus aureus* bacterial portrait

Staphylococcus aureus is the most significant human pathogen among the genus *Staphylococcus*. Its colonizing ability frequently allied to its extraordinary pathogenic power includes it amongst the best well characterized microorganisms.

1.1. *S. aureus*, historical perspective

After Robert Koch in 1878 recognized that different diseases were produced by distinct Gram-positive cocci with diverse bacterial arrangements (pairs, chains or clusters), Louis Pasteur and Alexander Ogston, a Scottish surgeon, described in 1880 the "small spherical bacteria" as a cause of pus from furuncles and abscesses and called them micrococci. However, it was Alexander Ogston, who demonstrated that the small spherical bacteria produced inflammation and suppuration and in 1882, named the clustered micrococci as "staphylococci" from the Greek *staphyle*, meaning bunch of grapes. In 1884, Anton J. Rosenbach, a German surgeon, provided the first taxonomic description of the genus *Staphylococcus* after the isolation of two species with different pigment appearance of the colonies: *Staphylococcus aureus*, from the Latin *aurum* for gold, and *Staphylococcus albus* (later named *Staphylococcus epidermidis*) from the Latin *albus* for white (163, 231).

1.2. Culturing and identifying staphylococci and *S. aureus*

The genus *Staphylococcus* is currently composed of 44 species and 24 subspecies (84), sharing common features: Gram-positive cocci with 0.5 to 1.5 μm in diameter, characteristically dividing in three successive perpendicular planes to form grape-like clusters, and having a unique cell wall peptidoglycan with multiple glycine residues in the interpeptide bridge, which renders them susceptible to lysostaphin. Staphylococci are nonflagellated, facultative anaerobes, glucose fermenting, catalase positive, oxidase negative, and tolerant to high saline concentrations with most species able to grow in the presence of 1.7 M of NaCl (231). These microorganisms are the hardiest of all non-spore-forming bacteria capable to grow in a range of temperature from 10º to 45ºC, with some strains

showing resistance to heat (withstanding 60ºC for 30 min) as well as to most disinfectants (231). The G+C content of *Staphylococcus* DNA is within the range of 30 to 38 mol percent, which makes the genus one of the members of the low G+C Gram-positive bacterial phylogenetic group (231).

The production of the enzyme coagulase remains one of the main tests to identify *S. aureus*, although two animal-specific coagulase-variable species have been described, *Staphylococcus intermedius* and *Staphylococcus hyicus* (163, 231). *S. aureus* forms typical golden yellow colonies, smooth, slightly risen and hemolytic on 5% sheep blood agar. *S. aureus* is the only *Staphylococcus* species that ferments mannitol anaerobically, and in complex media grows over a wide range of pH (4.8 to 9.4) and temperature (25 to 43ºC), showing a minimum doubling time of 30 to 40 minutes (231).

1.3. *S. aureus* as a human colonizer

S. aureus is a ubiquitous commensal and colonizer of the skin and mucosa of humans and animals that may turn into an important pathogen (188, 340). In humans, the primary ecological niche of *S. aureus* are the anterior nares (i.e, the vestibulum nasi), although multiple body sites can be colonized, such as the skin, perineum, pharynx and less frequently the gastrointestinal tract, vagina and axillae (254, 340).

In 1915, during the treatment of bacterial wound infections of soldiers from the World War I, Sir Almroth Wright, a British bacteriologist and immunologist, reported that bacterial wound infections probably had an endogenous source. However, it was Niels Danbolt, a Norwegian dermatologist and professor of medicine, who first reported, in 1931, the association between *S. aureus* nasal carriage and staphylococcal disease during the study of furunculosis (293, 349). Later, typing of *S. aureus* isolates brought additional evidence for the causal relation between *S. aureus* nasal carriage and infection, since nasal carriage strains showed the same genotype as infectious isolates. Nasal carriers have a three times increased risk of being infected with an endogenous *S. aureus* than non-carriers (340), and therefore decolonization attempts have been carried out in

order to reduce infection, cross-transmission and eradication of *S. aureus* reservoirs in nosocomial settings (5, 169).

Decolonization of the nostrils with mupirocin ointment and the skin with chlorohexidine gluconate soap has shown to be effective in preventing hospital associated *S. aureus* infections (28, 96). Nowadays, nasal mupirocin ointment is the first option for nasal carriage eradication.

Longitudinal studies distinguish three *S. aureus* nasal carriage patterns in healthy individuals: ~20% (range 12-30%) of individuals are persistent carriers, ~60% (range 16-70%) are intermittent carriers and ~20% (range 16-69%) are non-carriers (254, 340). However, a recent study stated that apparently, there are only two types of nasal carriers: persistent carriers and others, since intermittent carriers and non-carriers share similar *S. aureus* nasal elimination kinetics and antistaphylococcal antibody profiles (321). Persistent carriage seems to have a protective effect on the acquisition of other strains due to bacterial competition, is more common in children than in adults and many individuals change their pattern of carriage between the age of 10 to 20 years (164, 340). In contrast to intermittent carriers who usually carry different strains over time, persistent carriers are often colonized with a single strain over extended periods and with higher *S. aureus* loads that contribute to the enhanced risk of autoinfection and ease of dispersal in the environment (169, 327, 340). Cross sectional surveys of general healthy adult populations reported a total *S. aureus* nasal carriage rates ranging from 25-38%, depending on the study population, and usually rates of methicillin-resistant *S. aureus* (MRSA) carriage lower than 1.5% (164, 201, 302, 340).

Nasal colonization by *S. aureus* follows four main prerequisites: (i) contact of *S. aureus* with the anterior nares; (ii) *S. aureus* adherence to specific receptors in the nasal mucosa; (iii) overcome of host defenses and competition with the other resident microorganisms; and (iv) ability to propagate in the nose (340). Although the mechanisms leading to *S. aureus* nasal carriage are known to be multifactorial, including bacterial factors and interactions between bacterial organism and host, it seems that host characteristics substantially co-determine the *S. aureus* carrier state (183, 340).

Bacterial factors such as the wall teichoic acid (WTA), clumping factor B (*clf*B), the capsular polysaccharide (*cap*), the iron-regulated surface determinant IsdA, the surface protein SasG, autolysins and other virulence and adherence factors are thought to be inherent to the biological fitness and involved in the bacterial competition process that define which strain is able to colonize the host (254, 338, 341).

On the other hand, host factors are probably involved in the definition of carrier or non-carrier status, including the persistent or intermittent carriage. Studies on artificial colonization of individuals with known carriage status showed that intermittent carriers or non-carriers eliminate the colonizing strains whereas persistent carries maintain their original resident strain (230, 321). Inherent host factors such as race, sex, age, hormonal status in woman, physiological parameters as the glucose concentration in blood, anatomic alterations of the nares including the number and nature of bacterial adherence receptors, specific anti-staphylococcal components in the nasal secretions (e.g. lysozyme, lactoferrin, secretory leukocyte protease inhibitor, neutrophil alpha-defensins, beta-defensins or cathelicidins), certain types of histocompatibility antigens (e.g. HLA-DR3), polymorphism in genes codifying for glucocorticoid receptors, vitamin D receptor, Fc fragment of IgG, interleukine-4, C-reactive protein, the human complement cascade activator serine protease C1 inhibitor (C1INH V480M), and some additional acquired defects in immunity are potential determinants in carriage (34, 292, 317, 327, 340). However*, S. aureus* has proven to be able to specifically adapt to different niches in the human host (34).

Increased *S. aureus* carriage rates have been reported in certain patient groups: patients with diabetes mellitus (both insulin dependent and non-dependent), undergoing hemodialysis or continuous ambulatory peritoneal dialyses, rheumatoid arthritis, human immunodeficiency virus (HIV) infection or viral infectious of the upper respiratory tract, intravenous drugs addicts, patients with skin infectious and skin disease, obesity, and patients receiving repeated injections for allergies. Repetitive or long-term injury to the skin due to needle punctures seems to be a common risk factor for colonization in these patient

groups (254). Moreover, nasal screening in health care workers (HCW) revealed *S. aureus* carriage rates ranging from 25.7% to 67.2% (179, 288).

In addition to the enhanced carriage rates of hospitalized patients and HCW, veterinary staff, pet owners and farmers that have contact with pigs and poultry have been pointed recently as new risk groups for colonization, with rates of 5.6 to 12.3% of MRSA carriage (5, 184, 322).

1.4. *S. aureus* as a human pathogen

S. aureus is the most significant human pathogen in the genus *Staphylococcus,* capable of promoting disease when the natural skin barrier and mucous membranes are disrupted (183, 188). Of the approximately 2 million patients who acquire a health-care associated infection annually in the United States, around 292,000 have an infection associated to *S. aureus*, and of these, about 126,000 acquire an MRSA infection (172, 306).

S. aureus is responsible for a wide variety of infections, such as: (i) superficial lesions and skin infections (e. g. impetigo, folliculitis, carbuncles, mastitis, cellulitis); (ii) life threatening conditions (e. g. bacteremia, endocarditis, pneumoniae, meningitis, septicemia and osteomyelitis); and (iii) toxin-mediated diseases (e. g. scalded skin syndrome, toxic shock syndrome and food poisoning) (188, 306).

The success of the organism as a pathogen and its ability to cause such a wide range of infections depends on a complex interplay between *S. aureus* virulence determinants and the host defense mechanisms (10, 188).

1.4.1. Virulence determinants: structural key factors

S. aureus contains an extensive variety of virulence factors, including both structural and secreted products, that can be divided into functional categories according to its main role in the pathogenesis process: (i) attachment: factors involved in the adhesion of the bacteria to cells or extracellular matrices; (ii) evasion of host defenses: factors that prevent or reduce phagocytosis or that interfere with the function of specific antistaphylococcal antibodies or other particular host defense mechanisms; (iii) invasion or tissue penetration: factors

that specifically attack host cells and/ or degrade components of extracellular matrices; and (iv) maintenance in the host: proteins for utilization of host nutrients by the bacteria (13, 233).

S. aureus has numerous surface proteins, called “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that mediate adhesion to host tissue to initiate the infectious processes (108, 252).

S. aureus can form biofilms (slime) that are complex bacterial populations surface-attached and enclosed in a poly-N-acetylglucosamine matrix, enabling it to persist by evading host defenses and antibiotics (77). Furthermore, staphylococci avidly enter into the intracellular environment of endothelial cells, a protected milieu that promotes the formation of small-colony variants (SCVs) (188, 331). SCVs are sub-populations of *S. aureus* that exhibit a slow growth, distinct phenotype and pathogenic features that allow them to be hidden in host cells avoiding host defenses and antibiotics (331).

One of the *S. aureus* main defenses in evading the host immune system is the production of an antiphagocytic microcapsule, namely types 5 and 8 in clinical isolates, staphylokinase, staphylococcal complement inhibitor (SCIN) and *S. aureus* chemotaxis inhibitory protein (CHIPS) which are modulators of the immune system (93, 188, 274).

α-type phenol-soluble modulins (PSMα) are a recently described group of secreted staphylococcal peptides that have a remarkable ability to recruit, activate and subsequently lyse human neutrophils, thus eliminating the main cellular defense against *S. aureus* infection. A higher expression of PSMα was observed in *S. aureus* infections in the community (335).

Another two potential *S. aureus* virulence factors are a cluster of arginine catabolism (*arc*) genes that encode an arginine deiminase pathway, and *opp3*, which encodes an oligopeptide permease, both included in the arginine catabolic mobile element (ACME). While ACME is highly common in *S. epidermidis* (210), in *S. aureus* is almost exclusively found in a particular community associated lineage (USA300) (71).

1.4.2. Staphylococcal toxin determinants

Once the infection is established, *S. aureus* secretes a high number of toxins, including cytolysins, exfoliatins and superantigenic toxins (SAgs) that enable invasion, destruction of host tissues and spread to other sites (194, 258).

Cytolytic toxins, include α-, β-, γ- and δ-hemolysins, leukocidins (LukD-LukE and Luk M) and the Panton-Valentine leukocidin (PVL) that form beta-barrel pores in the cytoplasmatic membrane of cells of the host immune system, namely erythrocytes and leukocytes, promoting lysis (93, 194). While α-hemolysin was found to play a role in community acquired pneumonia, other cytolysins were found in a high percentage of *S. aureus* nosocomial isolates (32, 259). Due to its actual particular relevance, PVL will be described in more detail below.

Staphylococcal epidermolysins or exfoliative toxins, (ETA, ETB, ETD and ETC) are the major causative toxins of bullous impetigo and staphylococcal scalded skin syndrome (SSSS) (258).

Superantigen toxins are powerful immuno-stimulatory proteins that activate T-cells through the cross-linking of the T-cell receptor with the major histocompatibility complex (MHC) class II, inducing T-cell proliferation and massive cytokine production (14). These toxins include the staphylococcal enterotoxins (SEs), toxic shock syndrome toxin (TSST) and exfoliative toxins involved in SSSS (73). Staphylococcal enterotoxins have a notorious role in *S. aureus* food poisoning (177). Multiple superantigenic toxin genes are commonly found among *S. aureus*, namely associated with mobile genetic elements such as pathogenicity islands, prophages and plasmids (232). Recently, two studies on nasal and invasive *S. aureus* showed that the presence of different superantigenic and exfoliative toxin genes is strongly associated to *S. aureus* clonal lineages, rather than to invasiveness (136, 326).

1.4.3. Panton-Valentine leukocidin, a special role in infection?

PVL belongs to the family of “bicomponent synergohymenotropic toxins”, comprising two-component pore-forming toxins that act in synergy to lyse host cell membranes, namely mononuclear and polymorphonuclear cells (180). The two active proteins are designated S (from slow-eluted) and F (from fast-eluted) and

are encoded by two contiguous and co-transcribed genes, *lukF-PV* and *lukS-PV* (348), which are carried on temperate bacteriophages (153). PVL contributes to pathogenesis by promoting an exaggerated inflammatory response and injury to the host. The formation of pores on the cell membranes results in cell lysis and activation of neutrophils to release potent mediators of inflammation such as enzymes and cytokines (168, 202). PVL also appears to induce neutrophil apoptosis (101).

Although the role of PVL in dermonecrosis was established (58, 337), several studies comparing the virulence of PVL-positive and PVL-negative strains in animal infection models gave conflicting results about the exact function of PVL in pathogenesis (33, 72, 175, 332). Löffler et al. recently demonstrated that PVL acts differentially on neutrophils of various species, namely inducing rapid activation and cell death in human and rabbit neutrophils, but not in murine or simian cells, which could explain the controversial results (185). Diep et al. confirmed the role of PVL by postulating a model for the PVL-induced acute lung injury and lung inflammation based in a rabbit model of necrotizing pneumonia (70).

1.4.4. Virulence regulatory systems

Regulation of expression of the staphylococcal virulence factors plays a central role in pathogenesis, and at least, three major operons are involved: the *agr* (accessory gene regulator) (264, 312), the *sar* (staphylococcal accessory regulator) (44) and the *sae* (staphylococcal accessory element) loci (1). Additional regulatory systems also affecting virulence genes expression include: *arlR* and *arlS* (autolysis-related locus sensor) (94), *rot* (repressor of toxins) (280), *mgr* (multiple gene regulator) (189) and the staphylococcal alternative sigma factor, *sigB* (228).

To reduce undue metabolic demands, expression of virulence factors occurs in a coordinated mode: genes coding for surface proteins involved in adhesion and defense against the host immune system are down regulated during early stages of growth, whereas genes that encode secreted proteins are up regulated in the exponential phase. This shift in expression patterns is effected by

the *agr* system and translates a strategic switch from an early establishment phase to a late attack phase (234). The staphylococcal *agr* locus encodes a quorum-sensing (QS) system that works as a classical two-component signaling module (234). Allelic variations in the *agr B–D–C* region have resulted in at least four *agr* specificity groups in *S. aureus* (148).

It is important to note that not all *S. aureus* stains produce the same virulence factors, which in some cases could be clonally related (108). Although no evidence for hypervirulence was found (90), basically any *S. aureus* genotype can become a life-threatening human pathogen (197).

1.5. Has the era of *S. aureus* untreatable infections arrived?

Resistance to antibiotics arises either by mutation in chromosomal genes or by acquisition of foreign genes through horizontal gene transfer of mobile genetic elements (*e.g.* plasmids, transposons, insertion sequences, chromosomal cassettes) (20, 145, 261). Resistance can further spread vertically by dissemination of resistant clones (20).

Before the “antibiotic era”, approximately 90% of the *S. aureus* bacteremias were fatal (17, 261). In the early 1940s, with the introduction into clinical practice of the beta-lactam antibiotic penicillin G, the mortality rates decreased considerably (187). However, the increased use of penicillin resulted a few years later in the emergence of the first penicillin resistant *S. aureus* strains. Penicillin resistant strains produce a plasmid-encoded penicillinase that hydrolyses the beta-lactam ring of penicillin and by 1946 about 60% of the nosocomial isolates of *S. aureus* in the United Kingdom and United States were resistant to penicillin (6, 17, 145).

Records of the Danish Health Board registered the sequential introduction of several antimicrobial agents into clinical practice from 1945 to 1953 [penicillin, streptomycin, tetracycline and erythromycin] and reported a consecutive appearance of resistant *S. aureus* bloodstream isolates. By the late 1960s, the prevalence of penicillin resistance in the Danish nosocomial setting reached 85% to 90% (146).

In 1959, methicillin, a semi-synthetic beta-lactamase-resistant penicillin, initially called celbenine, was successfully introduced into clinical practice. Within two years, the first reports of methicillin resistant *S. aureus* strains were published (16, 147). By the end of the 1960s, multidrug-resistant MRSA became endemic in hospitals throughout Europe, Australia and India (59, 247, 276) and during the 1980s in the United States as well. Subsequently, MRSA isolates disseminated worldwide, becoming a major cause of infections in hospitals and the community (18, 60, 67, 162, 212, 226)

The hallmark of *S. aureus* as a pathogen is its capacity to acquire resistance mechanisms to virtually all antibiotics introduced into clinical practice (Table 1) (261). The increased use of vancomycin, a glycopeptide antibiotic to which MRSA were consistently susceptible, resulted in the emergence in 1996 of vancomycin intermediate *S. aureus* (VISA) and in 2002 of vancomycin resistant *S. aureus* (VRSA) strains (37, 132). Although initially VRSA were exclusively confined to the United States, two VRSA were later reported in the 2008 European Antimicrobial Resistance Surveillance System (EARSS) annual report (83).

Consequently, the launching of new antibiotics, such as quinopristin-dalfopristin, linezolid, daptomycin, tigecycline and telavancin was of great importance. However, resistance to most of these alternative antibiotics has already been reported (127, 129, 131, 314). Other vancomycin derivatives (dalbavancin, telavancin and oritavancin) as well as a modified carbapenem (razupenem), a novel fluoroquinolone (delafloxacin), oxazolidinone (radezolid) and cephalosporins (ceftobiprole and ceftaroline) seem to be valuable candidates to treat MRSA (24, 49, 57, 78, 239, 354).

Alternative approaches to the use of antibiotics have been proposed to treat MRSA infections: (i) modification of *S. aureus* genes associated to virulence to reduce infectivity; (ii) topical eradication of nasal carriage; (iii) strict infection control measures; and (iv) development of *S. aureus* vaccines (28, 75, 286, 325).

Table 1. *A*ntimicrobial agents' classes, mechanisms of action and resistance in *S. aureus.* Adapted from (87, 261).

Class	Mechanism of action	Resistance mechanism	Generic name (examples)
ß-Lactams (penicillins, cephalosporins, carbapenems)	Inhibit transpeptidation step in peptidoglycan synthesis; bind penicillin-binding proteins, stimulate autolysins	ß-lactamase; modified target (alteration of penicillin-binding protein)	Penicillin, Ampicillin, Cephalotin, Methicillin, Oxacillin, Cefepime, Ceftazidime
Aminoglycosides	Inhibit protein synthesis (30S)	Inactivation by aminoglycoside –modifying enzymes	Gentamicin, Kanamycin, Tobramycin, Streptomycin
Chloramphenicol	Inhibit protein synthesis (50S)	Target modification by acetyltransferase	Chloramphenicol
Daptomycin (cyclic lipopeptides)	Inhibit lipotheichoic acid biosynthesis; disrupt bacterial membrane	Mutations in multiple chromosomal loci (i.e. *mprF*, *yycG*, *rpoB*, and *rpoC*)	Daptomycin
Fluoroquinolones	Bind topoisomerase type II and IV	Efflux; mutation in topoisomerase II and IV genes	Ciprofloxacin, Moxifloxacin
Fosfomycin	Inhibit peptidoglycan synthesis; (inactivation of MurA)	Transporter mutation	Fosfomycin
Fusidic Acid	Inhibit protein synthesis (elongation factor G)	Target alteration; reduced influx	Fusidic Acid
Glycopeptides	Inhibit transglycosylation and transpeptidation steps in peptidoglycan synthesis by binding D-Ala-D-Ala	Gram-negative outer membrane; modified target (substitute D-Ala-D-Ala for D-Ala-D-lactate)	Teicoplanin, Vancomycin
Glycylcyclines	Inhibit protein synthesis (30S)	Efflux pumps system	Tigecycline
Macrolides/lincosamides	Inhibit protein synthesis (50S)	Methylation of target; efflux; enzymatic inactivation	Azithromycin, Erythromycin, Clindamicin
Mupirocin	Inhibit protein synthesis (isoleucyl-tRNA synthetase)	Target modification	Mupirocin
Linezolid (Oxazolidinones)	Inhibit protein synthesis (50S)	Mutations in 23S rRNA genes; methylation of 23S rRNA	Linezolid
Rifampicin	Binds ß-subunit of bacterial RNA polymerase	Mutation in RNA polymerase	Rifampin
Streptogramins	Inhibit protein synthesis (50S)	Inactivation of antibiotics by hydrolysis or acetylation; methylation of 23S rRNA	Quinupristin-Dalfopristin
Tetracyclines	Inhibit protein synthesis (30S); disrupt bacterial membrane	Ribosome protection; efflux system	Tetracycline
Sulfonamides and trimethoprim	Inhibit enzymes responsible for tetrahydrofolate production	Mutations alter affinity for target enzymes	Trimethoprim, Trimethoprim-Sulfamethoxazole

1.5.1. Resistance to topical antibiotics

Fusidic acid has been effectively used as a topical agent in *S. aureus* skin and soft tissue infections (282). However, long-term therapy is suspected to be responsible for increasing resistance (263). Fusidic acid resistance genes (*fusB*, *fusC* and *fusD*) were prevalent among European staphylococcal isolates in 2008 (36). The possible emergence of *fusA* and *fusE* mutations by extensive and continuous use of this antibiotic is of major concern in the current "era" of CA-MRSA, therefore the combination with other agents, such as rifampin is strongly recommended (76).

Mupirocin has been employed successfully as a topic nasal ointment to decolonize MRSA carriers and to treat superficial skin infections. While both high-level (generally plasmid mediated and encoded by the *mupA* gene) (134) and low-level (associated to point mutations in the chromosomal *ileS* gene) (9) mupirocin resistance was reported, the prevalence of resistant isolates is widely variable among different populations, but remains generally low (150). However, institutions that are considering the implementation of widespread mupirocin use for nasal decolonization are encouraged to reflect on the resistance issues so as not to compromise the management of MRSA carriers (251).

1.5.2. Resistance to methicillin: *mecA* gene and SCC*mec*

The central element of methicillin resistance is the *mecA* gene that encodes a unique penicillin binding protein PBP2a (or PBP2') which has low affinity for beta-lactam antibiotics and can function as a substitute of the native staphylococcal PBPs that could be inactivated by beta-lactams (62). Besides the undoubted role of *mecA* as the primary determinant of methicillin resistance, mutations in additional genes termed *fem* (factors essential for expression of methicillin resistance) or *aux* (for auxiliary) can also affect the expression of resistance (21).

The *mecA* gene has been found as part of a large mobile genetic element, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) which may contain additional resistance genes (143, 157).

The SCC*mec* elements are characterized by the presence of (i) terminal inverted and direct repeats; (ii) site-specific recombinases designated as cassette chromosome recombinases (*ccr*) that allow the mobilization of the cassette; (iii) a *mecA* gene complex; and (iv) a specific integration site sequence (ISS) (141). SCC*mec* integrates in the chromosome of susceptible strains at a site specific location near the origin of replication, the *orfX*, a gene of unknown function (157). The classification of SCC*mec* is based on binary combinations of the *mec* complex and the *ccr* allotype (Figure 1). The *mec* complex is composed of the *mecA* gene, its regulatory genes (*mecR1* and *mecI*) and associated insertion sequences. The *ccr* complex is composed of the *ccr* gene(s) and surrounding open reading frames (ORFs). So far, four classes of *mec* complex (A, B, C [C1 and C2] and E) and five *ccr* gene complexes (types 1 to 4, carrying two genes, *ccrA* and *ccrB* with four allotypes: *ccrAB*1, *ccrAB*2, *ccrAB*3, *ccrAB*4, and type 5 carrying a single allotype, *ccrC* with five alleles: *ccrC*1 allele 1 to 4 and *ccrC*1 allele 8) have been described in *S. aureus*, corresponding to eight SCC*mec* types (I to VIII) (Figure 1): SCC*mec* type I (class B *mec* complex, *ccrAB1*), SCC*mec* type II (class A *mec* complex, *ccrAB2*), SCC*mec* type III (class A *mec* complex, *ccrAB3*), SCC*mec* type IV (class B *mec* complex, *ccrAB2*), SCC*mec* type V (class C2 *mec* complex, *ccrC*), SCC*mec* type VI (class B *mec* complex, *ccrAB4*), SCC*mec* type VII (class C1 *mec* complex, *ccrC*), and SCC*mec* type VIII (class A *mec* complex, *ccrAB4*) (22, 121, 141-144, 178, 190, 242, 355). An additional *mec* complex class D was described in *Staphylococcus caprae* but so far not found in *S. aureus* (156).

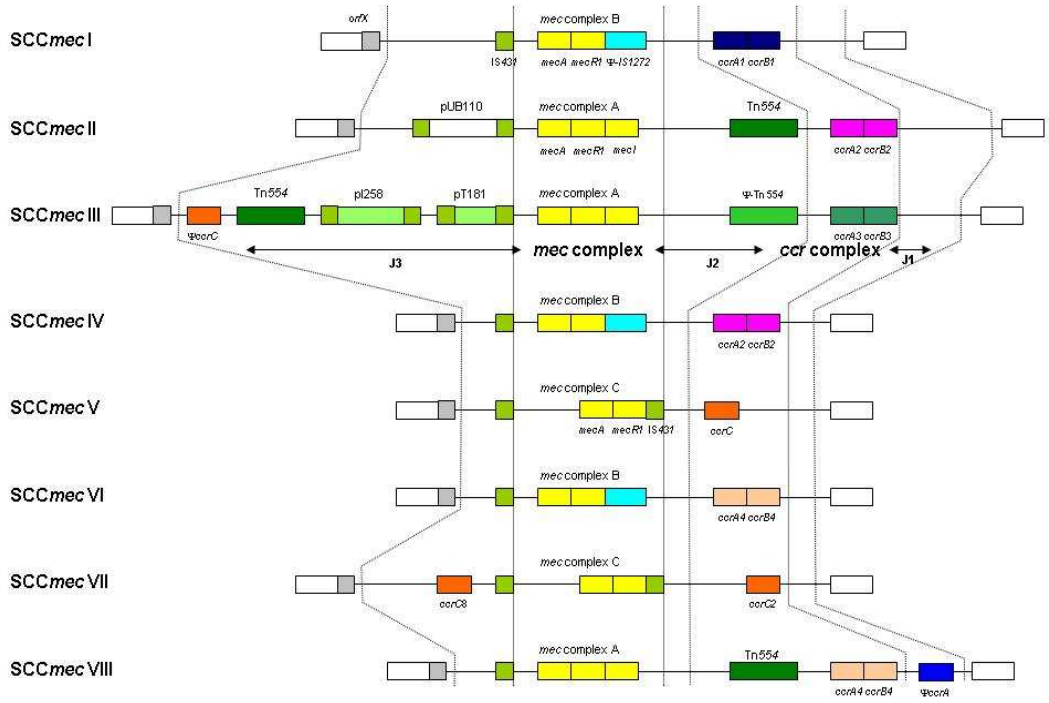

Figure 1. Genetic organization of SCC*mec* types I–VIII. Figure is not to scale. Reprinted with permission from (87).

In addition to the *mec* and *ccr* complexes, SCC*mec* elements contain three J regions (J1, J2 and J3) that stand for "junkyard" or "joining regions" (Figure 1), composed by non-essential components and in some cases additional antimicrobial resistance determinants. These J regions may include polymorphisms or variations within the same combination of the *mec* and *ccr* complexes, which is used to define SCC*mec* subtypes (141). So far, 26 subtypes of SCC*mec* have been described among the eight major types. Of major interest is the subtyping of SCC*mec* type IV, the smallest, most mobile and most variable structural type of SCC*mec*, present not only in the overwhelming majority of emergent community-acquired MRSA (CA-MRSA), but also in some international nosocomial clones (64, 344). Ten SCC*mec* type IV subtypes have been described so far with variations in the J1 region (Figure 2), which allows a more specific

epidemiological characterization of MRSA collections with high percentage of isolates carrying a type IV cassette (23, 141, 203).

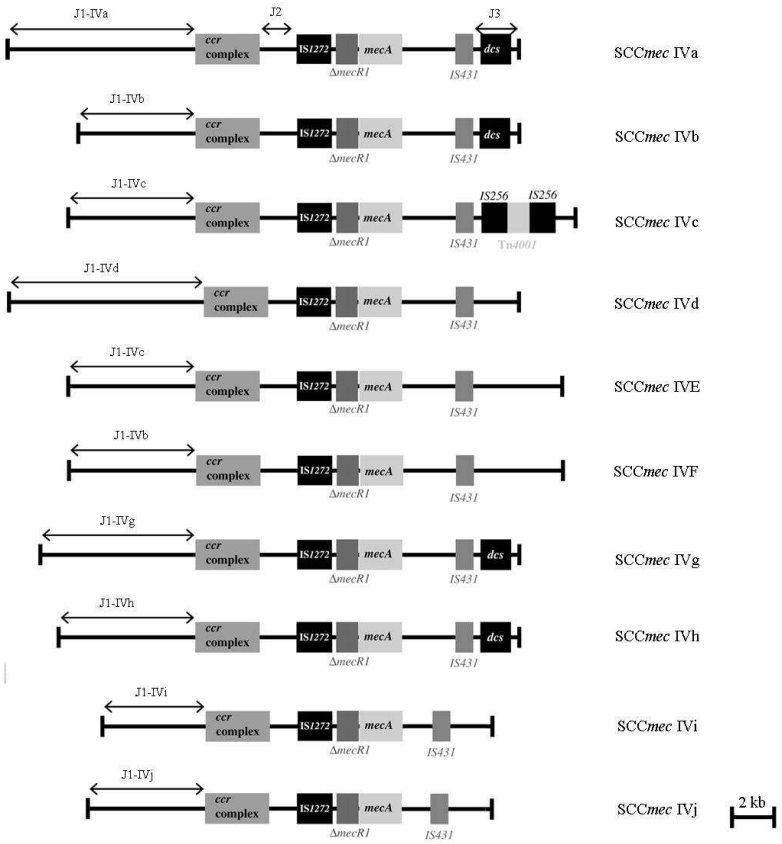

Figure 2. Genetic organization of the subtypes of SCC*mec* type IV. Adapted from (23, 203).

A recent standardization of the SCC*mec* classification and nomenclature, is available at http://www.sccmec.org, and has been proposed to accommodate the increasing number of types, subtypes and variants (47, 141).

The origin of the *mecA* and SCC*mec* is still a matter of debate. No homologue of the *mecA* has been found in MSSA strains and it has been assumed that it is an exogenous gene that originated in coagulase-negative staphylococci (CoNS) (11, 63). Couto et al. evidenced that the *mecA* determinant may be a housekeeping gene in a fully beta-lactam susceptible *Staphylococcus sciuri* (56) which is a frequent colonizer of the skin of many domestic and wild animals and is one of the most abundant staphylococcal species on Earth (163). The *mecA* found

in *S. sciuri* has 88% homology on the amino acid level to the MRSA gene (350). Moreover, introduction of the *S. sciuri mecA* into an MSSA resulted in increased resistance to methicillin together with the detection of a protein homolog to PBP2a (351). SCC*mec* elements are widely found among CoNS which constitute another line of evidence of the role of CoNS as reservoirs for the *mecA* acquisition by *S. aureus* (100, 158, 213). A recent study suggests *Staphylococcus fleurettii*, a commensal bacterium of animals, as a probable intermediary in the evolution of the *mecA* gene. Although the *mecA* in *S. fleurettii* was not associated to SCC*mec*, it is located in a chromosome locus with an identical sequence to that of the *mecA*-containing region of SCC*mec* and therefore is speculated that SCC*mec* was assembled by adopting the *S. fleurettii mecA* gene and its surrounding chromosomal region (315).

1.6. *S. aureus* costs to individuals and society

S. aureus infections present financial issues not only for the individual, but for the society as well, due to prolonged hospital stays, costly medical procedures, and medications. *S. aureus* infection was reported for 0.8% of all hospital inpatients, and was associated to 292,045 days of hospital stay per year in the United States hospitals in 2000-2001. Inpatients with *S. aureus* infection present, on average, a length of hospital stay three times higher (14.3 vs. 4.5 days; $P<0.001$), total charges three times higher ($48,824 vs. $14,141; $P<0.001$), and the risk of in-hospital death five times higher (11.2% vs. 2.3%; $P<0.001$) than non-infected inpatients (229).

Moreover, various studies showed that, compared to MSSA, MRSA infections cause longer hospital stays, higher costs and higher mortality (19, 53, 55). It has been estimated that MRSA hospital costs in the United States range between $1.5 and $4.2 billion at the national level, and the costs to society between $17 and $30 billion (109). In a recent study in Northern Europe, the total additional costs due to an MRSA outbreak in surgical and internal medicine units were calculated in €386,062 (70% for screening and 25% for contact isolation) (154).

The increasing burden of *S. aureus* infections worldwide conducted to the development of infection control strategies such as the "Search and Destroy policy" in countries of Northern Europe (The Scandinavian countries and The Netherlands). Controlling MRSA is expensive, even though recent studies provided evidence that the strict application of these "Search and Destroy" measures can effectively control an epidemic of MRSA colonization and infection, preventing 36 cases of MRSA bacteremia per year, which resulted in annual savings of €427,356 for the hospital and ten lives per year (237, 325).

2. Bacterial typing: tools for fighting *S. aureus*

2.1. Why typing of *S. aureus*?

Typing is roughly defined as the attribution of labels to bacterial isolates, and is greatly facilitated by the increasing variability of typing methods currently available (319). The identification of the pathogen distribution and strain relatedness is essential for the determination of the epidemiology of nosocomial infections. Therefore, *S. aureus* typing is of major importance in: (i) outbreak situations, where the identification of the causative agent, the identification of bacterial reservoirs and transmission routes, increase the efficacy of control; (ii) surveillance studies, allowing an early warning of potential outbreaks by the detection of bacterial clusters, and determination of the clonal nature of bacterial populations; (iii) evolutionary studies, determining the *S. aureus* population structure and genetics, providing important clues about evolutionary trends of the bacterial pathogen as well as the emergence of clinically relevant strains with increased resistance or virulent content (290, 319, 339).

In addition to the critical role of typing in outbreak investigations and epidemiological surveillance of *S. aureus* infections, it also contributes to elucidate the progress of infection in single patients or even identifies the exogenous or endogenous microflora origin of the infection causative agent (290, 319).

Typing could be considered as comparative, usually used in outbreaks situations, or definitive including the creation of strain collections available for

comparative assessment between laboratories (319). *S. aureus* typing can be undertaken at different levels: (i) locally, usually for outbreak investigations in a hospital laboratory; (ii) regionally or nationally, in reference laboratories for more robust surveillance studies; (iii) internationally, through collaborative networks, to define or survey the worldwide dissemination of epidemic clones (319).

Different typing methods have been used at each level, based on *S. aureus* phenotypic and/ or genotypic features. However, no single typing method has proven to be solely sufficient to characterize a collection of isolates (299, 339).

2.2. The value of a typing method

Every typing method should meet performance and convenience criteria (319). Performance criteria include: (i) stability, meaning that the method should assess a marker that remains constant during the study period; (ii) typeability, translating the ability of a method to assign a type to all the isolates tested; (iii) discriminatory power, referring to the ability of the method to differentiate two epidemiologically unrelated isolates from the population; (iv) epidemiological concordance, i.e. the agreement between the typing results and the epidemiological information of the isolates; (v) and reproducibility, meaning that a typing result for an isolate should be identical in a different temporal and/ or spatial occasion. Convenience criteria should also be taken into account, namely feasibility, flexibility, rapidity and ease of use, highly related with the accessibility and cost inherent to the equipment and reagents used. The choice of a typing method depends not only in performance and convenience criteria, but also on the question to be addressed, the specific epidemiological situation and the resources available (290).

Recently, with the study of large collections of isolates, the amenability to computerized analysis as well as an easy incorporation of typing results into electronic databases are key points for choosing a typing method (319). Moreover, new *S. aureus* typing methods, making use of new technologies, or variation of well established methods, have been developed in order to meet these requisites. However, such methods require validation in terms of sensitivity and specificity, which is usually accomplished by comparison with well established methods (267).

The best approach is to evaluate the congruence in a quantitative way. Recently, Carriço and coworkers proposed a framework of measures for the quantitative assessment of correspondences between different typing methods for *Streptococcus pyogenes* (35), which were already applied to *S. aureus* and *S. epidermidis* data (88, 209). This Comparing Partitions framework included the Simpson's index of diversity, the adjusted Rand's index and the Wallace coefficients which could be assessed online (http://darwin.phyloviz.net/ComparingPartitions). The Simpson's index of diversity (289) associated to confidence intervals measures the probability of two isolates, randomly sampled from the population, to belong to two different types (115, 139). The adjusted Rand's index (138) quantifies the global agreement between two methods, whereas the Wallace coefficient (334) indicates the probability that two isolates classified as the same type by one method are also classified as the same type by another method. The use and interpretation of the congruence measures may be improved by the estimation of suitable confidence intervals. Therefore, the Comparing Partitions web-tool was updated with 95% confidence intervals for Wallace and adjusted Rand coefficients, reinforcing the role of these coefficients in generating maps of type or subtype equivalence and allowing an objective comparison between typing methods (285).

2.3. Phenotypic based methods

The earliest methods used to identify and type bacterial pathogens were based upon their phenotypic characteristics. Biotyping, antimicrobial susceptibility testing, phage typing, whole-cell protein electrophoresis, zymotyping, multilocus enzyme electrophoresis (MLEE), capsule typing and serotyping are some of the methods used in phenotypic characterization of *S. aureus* (290, 296). However, since phenotypic expression depends on environmental conditions, some of the performance criteria are not always full field. Therefore, only a small set of phenotypic methods are still currently used.

Phage typing

Phage typing classifies the strains based on the pattern of resistance or susceptibility to an approved set of bacteriophages by the International Subcommittee on Phage Typing of Staphylococci (26, 250). Currently 23 standard phages are applied to an agar plate covered with the test isolate, at the Routine Test Dilution (RTD), the highest dilution giving confluent lysis of the propagating strain, or at a 100 times concentration (100XRTD) when the isolates are nontypeable with RTD, in order to produce areas of lysis (*i. e.* plaques) (265). Since not all *S. aureus* isolates are lysed by the same phages, typical phage types could be defined. Some additional locally isolated phages are usually added to the approved international set to increase the discrimination of isolates (61).

For decades, phage typing was the standard method for *S. aureus* typing. However, some drawbacks such as limited typeability and reproducibility, variable discrimination, and technically demanding laboratorial efforts (preservation of stocks of phages and propagating strains) limited its use in routine (339). Therefore, phage typing is nowadays rarely used and in complement to other molecular methods in epidemiological studies (61, 343).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing, or antibiogram, determines the pattern of resistance or susceptibility of an isolate, *in vitro*, to a panel of antimicrobial agents. This method is routinely performed in clinical laboratories using either disk diffusion methods or broth microdilution in an automated way (290). Microdilution testing provides a quantitative measure of the minimal inhibitory concentration (MIC) defined as the lowest concentration of the antimicrobial agent that inhibits the growth of the microorganism, while the disk diffusion provides a measure of the growth inhibition halo from a defined concentration of antibiotic diffused in the media by a paper disk. Both methods follow well defined guidelines essential for standardization and interlaboratory reproducibility, such as the ones of the Clinical Laboratory Standards Institute (CLSI) (50).

Clinically, the antibiogram has immediate usefulness to guide antibiotherapy (319). In epidemiological studies, it has limited value, since isolates that are not genetically and epidemiologically related may have the same susceptibility pattern due to the acquisition or loss of mobile genetic elements carrying the same resistance genes, or even spontaneous point mutations driven by the high antibiotic pressure found in health care settings (290, 347). In contrast, the pattern of susceptibility was shown to be an excellent phenotypic marker for the identification of two distinct clones of MRSA in a Portuguese hospital (7).

2.4. Genotypic based methods

The introduction of molecular typing in epidemiological hospital surveillance is well-established and is proven to be cost-effective (8, 290).

Genotypic or molecular typing is a means of sampling the bacterial genome by assessing its variability in terms of composition, overall structure or precise nucleotide sequence. Basic genetic analysis of molecular events associated with pattern variation (e.g. deletions, insertions and mutations) is usually the best approach for the determination of strain relatedness. Molecular typing presumes that: (i) isolates from an outbreak have the same or highly related genotype and derive from a common ancestor or clone; and (ii) epidemiologically unrelated isolates have different genotypes (290).

DNA-based typing methods have significant advantages over phenotypic methods concerning typeability, discrimination, reproducibility, rapidity and ease of use. A wide variability of genotypic methods have been used for *S. aureus* typing (290, 296), fundamentally based on: (i) the Polymerase Chain Reaction (PCR) technique, (ii) endonuclease restriction to create band pattern fingerprints, (iii) arrays technology, and (iv) DNA sequencing.

(i) Despite automation attempts, traditional PCR-based strategies such as repetitive sequence based (rep-) PCR (323), random-amplified polymorphic DNA (RAPD) (4, 318) or single locus amplification of the coagulase gene (165), have been dumped for the identification and comparison of *S. aureus* isolates mainly due to lack of reproducibility, standardization and portability of results (65, 279). Other PCR based methods have been developed, such as

the restriction-modification (RM) test, based on three PCRs targeting the SauI RM system specificity subunit genes (*sau1hsdS1* and *sau1hsdS2*), which coupled to the rapidity of real time PCR, was reported as a simple, inexpensive and accurate method to identify *S. aureus* lineages (51). Also, Loughman et al. used a real-time reverse transcriptase PCR to detect the expression of virulence genes directly from *S. aureus* cells (186).

(ii) The endonuclease fragment restriction followed by hybridization with DNA probes was the basis for ribotyping (27), binary typing (324) and the study of polymorphisms using gene specific probes for *mecA* and Tn*554* (4, 171) effectively applied to the epidemiological study of *S. aureus* collections, but currently almost dismissed.

Methods such as the amplified fragment length polymorphism (AFLP) or the recent high-throughput modification (ht-AFLP) combine PCR with fragment restriction analysis to produce fingerprints for both MRSA and MSSA typing and population structure definition (197, 281).

(iii) Techniques such as microarray technology have been widely used in the evaluation of genetic variation of *S. aureus* lineages (174, 181, 216), characterization of community and hospital MRSA clones (166, 215), and detection of resistance, virulence and epidemicity markers (173, 214, 357).

(iv) Although full genome sequencing is still laborious, time demanding and expensive, low cost high throughput sequencing technologies have been developed. New comparative genomic approaches have been used to assess single nucleotide polymorphisms (SNP) and compare significant fragment mutations in relatively large collections of *S. aureus* isolates for evolutionary studies and geographical spread of specific clonal populations (128, 235, 236). Moreover, the tracking of *in vivo* evolution of multidrug-resistance in *S. aureus* isolates recovered periodically from the bloodstream of a patient undergoing chemotherapy with vancomycin and other antibiotics was assessed by whole-genome sequencing (225). A complete genome sequencing and further genome comparison was recently performed by Holden el al. for an unusually high transmissible MRSA isolate (135).

(v) Analytical technologies such as the mass spectrometry (MS) or microfluidics have been used as a means of automation, rapidity and throughput increasing on *S. aureus* identification and characterization. Pathogen profiling and identification of virulence and resistance genes was carried out coupling PCR to electrospray ionization-MS (EIS-MS) (118, 346). Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) or its surface enhanced variation (SELDI-TOF-MS) performed well in the identification of staphylococci, detection of the staphylococcal PVL gene and discrimination between MRSA and MSSA starting in intact cells (25, 79, 353). A new genomic typing technology, direct linear analysis (DLA) performed on microfluidic chips, was successfully applied to the molecular typing of *S. aureus* with a high discriminatory power, comparable to that of PFGE (342).

Although many molecular methods have been developed for the characterization of *S. aureus*, some were restricted to local or regional studies, and only a few are currently well-accepted and generally adopted: (i) pulsed-field gel electrophoresis (PFGE); (ii) multilocus sequence typing (MLST) (iii) *spa* typing; (iv) staphylococcal chromosome cassette *mec* (SCC*mec*) typing and (v) multilocus variable tandem repeats (VNTR) analysis (MLVA). Since these methodologies have been extensively used in the present work, a detailed description of each one will be provided.

PFGE

Pulsed-field gel electrophoresis (PFGE) was first described in 1984 as a tool for examining the chromosome of eukaryotic organisms (284). Afterwards, it has proven to be a highly effective molecular technique for many bacterial species including *S. aureus* (304). The method is based on the digestion of bacterial DNA with restriction endonucleases with relatively few recognition sites, generating 10 to 30 restriction fragments, which are further resolved as a pattern of distinct bands (303). Contrary to conventional electrophoresis, the electric field is applied in pulses that alternate in orientation, allowing the separation of the DNA fragments that range from 10 to 800 kb in length (284, 303). PFGE is still regarded

as the “golden standard” method in *S. aureus* typing, due to its high discriminatory power, reproducibility and high typeability, even for the recent “livestock-associated” sequence type 398 isolates that due to DNA methylation are insensitive to the conventionally used SmaI enzyme, and must be digested with the neoschizomer Cfr9I (29, 86). Random genetic events, including point mutations, insertions and deletions of DNA, may be detectable in PFGE patterns, which made the method suitable for comparisons of highly related isolates and hence for local outbreak epidemiologic analysis and for *S. aureus* surveillance studies at regional or national levels (105, 299, 304). However, PFGE requires relatively expensive equipment, is time and technically demanding, has some limitations in interlaboratory portability and uniform nomenclature (105, 299, 320). Consensus guidelines for correlating variations in restriction profiles with epidemiological relatedness, as well as harmonization protocols and laboratory networks have been proposed to overcome the referred drawbacks (48, 195, 223, 304).

MLST

Multilocus sequence typing (MLST) was originally developed to identify hypervirulent lineages of *Neisseria meningitidis* (191) and was then extended to a variety of other pathogens, including *S. aureus* (81). The method is based on the sequencing of internal fragments of housekeeping genes. For *S. aureus*, seven housekeeping genes were included in the MLST scheme: *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* (81). Fragments of 450-500 bp in size are used since they can be accurately sequenced on each strand with a single pair of primers and provide sufficient variation in the population (294). For each gene, sequences that differ even at a single nucleotide are assigned as different alleles. The combination of the allele at each of the seven loci defines the allelic profile, which unambiguously identifies the sequence type (ST) of each isolate. In *S. aureus* new alleles are most probably due to point mutations rather than recombination events (90, 294).

The major advantages of MLST are the portability and reproducibility of the sequence data. A freely accessible database is available on the internet (http//:www.mlst.net) allowing the assignment of the alleles and STs as well as the

deposit and comparison of the DNA sequences. The eBURST algorithm (from Based Upon Related Sequence Types) (91) is also freely available (http://eburst.mlst.net/), which allows the definition of evolutionary relationships between different STs by identifying groups of closely related genotypes, the clonal complexes (CC). CC emerges from a founding genotype that increases in frequency to become the predominant clone and gradually diversifies into other related genotypes (90, 295). If one, two or three of the seven loci are changed, single-, double- or triple-locus variants (SLV, DVL, or TLV) are generated, respectively.

MLST shows moderate discriminatory power for local epidemiological typing, which associated to the time and cost, compromises its use as a *S. aureus* primary typing tool (299). Since the variation in housekeeping genes indexed by MLST accumulates relatively slowly (294), this method is suitable for long-term epidemiological and population evolutionary studies (59, 81, 82, 90).

spa **typing**

spa typing, a single-locus sequence typing technique developed by Frenay et al., relies on the determination of sequence polymorphisms in the variable X region of *S. aureus* protein A gene (*spa*) (97). The polymorphic X region consists of a variable number of tandem short sequence repeats (SSR) located in the 3' coding region of the C-terminal cell wall attachment sequence of protein A (116). The SSR are usually 24 bp length, although 21, 27 and 30 bp repeats were also described (266). This X region is flanked by well conserved sequences permitting the annealing of primers and the subsequent amplification and direct sequencing of the entire region (2, 287). The high diversity within the SSR region is attributed to point mutations, deletions, duplications and insertions of repetitive units (151).

To each different repeat is assigned an alpha-numerical code, and the order of specific repeats defines the *spa* type. Two different nomenclature systems were developed, which made the comparison of published *spa* data difficult (126, 170). A recent software package, StaphType (Ridom GmbH, Würzburg, Germany) appeared as a valuable tool to overcome this drawback using the RIDOM nomenclature (126). The resulting sequence chromatograms are analyzed and

further synchronized to an online database, the SpaServer (http://spaserver2.ridom.de/index.shtml), for the assignment of the *spa* repeats and *spa* types (126). The SpaServer is one of the largest databases for *S. aureus* typing, curated by the European SeqNet network (http//:www.seqnet.org) that includes 50 laboratories from 27 European countries and provides nomenclature and epidemiological data (98, 99).

Despite being a single-locus typing method, *spa* typing is robust enough to cover genetic variations that accumulate at different evolutionary clock speeds (micro- and macrovariation) and therefore may be used for outbreaks, local and global epidemiological and population based studies (170, 298). It has become one of the primary typing methods for *S. aureus*, mainly due to its high throughput, ease of performance, shorter time comparatively to MLST, full portability of the data and straightforward interlaboratory comparison and reproducibility (2, 98, 298). A surveillance method based on *spa* sequencing and automated alerts, was useful as an early warning system to prevent hospital outbreaks (199).

Coupling the algorithm Based Upon Repeat Pattern (BURP) to the StaphType software enabled the grouping of related *spa* types into clonal lineages and the study of long-term evolution of *S. aureus* populations (200). Several studies demonstrated a good correlation between *spa* type clonal groups of MRSA and groups generated by other methods, namely PFGE types and MLST clonal complexes generated by eBURST (52, 120, 170).

However, as a single locus based method, *spa* typing showed some limitations in the differentiation of particular genetic lineages, namely CC1/CC80 and CC8/CC30/CC45 (298). Combinations of additional genetic markers, such as SCC*mec* for MRSA and lineage specific virulence genes for MSSA, are useful to increase the discriminatory power of the method (120, 298).

Recently, Grundmann et al. used *spa* typing to map the geographic distribution of MRSA and MSSA clones causing invasive infections in Europe and made available an online mapping platform for geographic tracking of strains/clones with particular public health importance (http://www.spatialepidemiology.net/SRL-Maps/) (113).

SCC*mec* typing

SCC*mec* typing has been recognized as an addendum to the MRSA clonal characterization, which is currently defined by the combination of the MLST sequence type (ST) and the SCC*mec* type (82, 272).

Since 2002, that a variety of PCR based methods have been described to determine the structure of the different SCC*mec* types harbored by MRSA. The SCC*mec* typing methodology inquires specific loci in the mobile genetic element by conventional or multiplex PCR. Oliveira et al. developed the first multiplex PCR assay that detects the *mecA* gene and eight loci scattered trough the entire cassette, resulting in specific band patterns for SCC*mec* types I-IV (241). This strategy misclassifies SCC*mec* types V/ VI and types III/ IV, respectively, since the former types were not described at the time of this multiplex design. In order to overcome the drawback, an update of the method was later published, allowing the association of SCC*mec* types I-VI to specific amplification patterns of 2-5 bands (204).

Alongside to the Oliveira et al. PCR multiplex, Okuma et al. developed a strategy that uses conventional PCR assays to determine the *mec* complex and *ccr* genes structure, where different combinations correspond to different SCC*mec* types (240).

Although several other multiplex strategies were proposed to overcome the limitation of these first strategies in the non identification of the new types V-VIII and subtypes (31, 167, 356), they were not extensively adopted since they lack discriminatory power, are laborious, time consuming and not practicable for routine typing.

In 2006, a different approach was proposed based on the sequencing of an internal fragment of the *ccrB* gene using a single pair of degenerated primers (243). An online database (http//:ccrbtyping.net) permits the user to query his own sequences, assign a *ccrB* allele and determine the most probable SCC*mec* type, as well as tracing the SCC*mec* elements within the MRSA population based on the stored sequence data (244). This tool has the advantage of being easily integrated into the *spa* typing or MLST protocols for routine typing.

The increasing number of MRSA infections in the community made the typing of SCC*mec* IV and its variants a major concern. Therefore, Milheiriço et al. developed a multiplex PCR assay based on the detection of polymorphisms in the J1 region to identify SCC*mec* type IV subtypes (IVa to IVh) (203).

The best SCC*mec* typing strategy depends on the purpose of the study. In this context, de Lencastre et al. (62) proposed a flexible and feasible SCC*mec* typing scheme including three sequential steps: 1) *ccrB* typing (243, 244); 2) SCC*mec* multiplex PCR, updated version (204); and in the cases of type IV cassettes 3) SCC*mec* IV multiplex PCR (203). In addition, a recent report of the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) proposed some guidelines for the classification, nomenclature and definition of new SCC*mec* types (141).

So far, no single multiplex PCR strategy can include the 22 out of 34 binary targets necessary to properly genotype all 46 SCC*mec* variants described by Stephens et al. (297). The continuous emergence of new SCC*mec* structures and variants requires a permanent update of the existing schemes or the design of new ones.

MLVA

The genome of *S. aureus* harbors a variety of loci of repetitive DNA, which may contain variable repeated units among strains and in many occasions constitutes a strain-defining parameter (182). Analysis of variable number of tandem repeats (VNTR) has been extensively used for typing clinical isolates of several bacterial species, including *S. aureus*, since a limited number of loci provides an overview of diversity within a bacterial population (182, 329). For this purpose the study of VNTRs was based in two interrelated basic methodologies: i) the production of multiple band patterns by multilocus VNTR fingerprint (MLVF) assays and ii) the determination of the number of DNA repeats per loci forming a string of numbers that characterizes each strain, the multilocus VNTR analysis (MLVA) (257, 279, 329).

Sabat et al. in 2003, proposed an MLVF method that discriminates different *S. aureus* clinical isolates based on the analysis of five (*sdr*, *clfA*, *clfB*, *ssp*, and

spa) tandem repeat loci (278, 279). The method has been extensively used as initially described or with slight variations such as the addition of virulence genes containing repeated units such as *fnbA*, *fnbB* and *can* (95, 103, 193, 198, 222, 255, 268, 279, 307). The discriminatory power and clustering capability similar to PFGE, made the MLVF method reliable for short-term epidemiological studies (193, 307). However, the fact that the data are interpreted by band patterns comparison in agarose gels makes the results inaccurate, not easily comparable between laboratories and not prone to be introduced in international databases (257, 279).

MLVA was first applied for *S. aureus* genotyping by Hardy et al. (123, 125). The method, designated staphylococcal interspersed repeat unit (SIRU) typing, relies on PCR amplification of seven loci of repetitive DNA and on the determination of the size of each amplicon. To each of the seven loci is attributed the respective number of DNA repeats, generating a code of seven numbers that characterizes each strain and corresponds to the allelic profile. This numerical allelic profile makes the SIRU data amenable to interlaboratory comparisons and database management, comparable to MLST. The strict methodology and slight modifications have been applied to nosocomial outbreaks and to a restricted European collection of contemporary *S. aureus* isolates (102, 122-124, 140).

Recently, two additional MLVA schemes were published, including new VNTR markers as well as some of the loci included in the previous referred assays (257, 283). Schouls et al. described a robust and portable MLVA system including eight loci amplified in two multiplex PCR reactions, followed by an accurate sizing of fluorescently labeled amplicons on an automated DNA sequencer. The validation of the method showed 100% typeability and the clustering was similar to *spa* typing or PFGE grouping (283). The MLVA-14 proposed by Pourcel el al. included two complementary panels totaling 14 VNTRs providing an easy and extremely informative genotyping assay with strong phylogenetic content and high congruence with MLST and *spa* typing (257). This method was successfully used in a longitudinal survey of *S. aureus* infections in 143 cystic fibrosis patients (333).

As a PCR based technique, MLVA is neither expensive nor time consuming, and is open to automation and portability of the data, which makes it

amenable for routine laboratories (95, 198). The adoption of capillary electrophoresis equipment, DNA sequencers and automated DNA sizing systems may improve the accuracy of the method (196, 329).

The major drawbacks of the MLVA methodology are the lack of standardization, since no consensus is achieved about the most informative VNTRs to be used. In order to overcome this disadvantage two MLVA databases are nowadays freely available.

Pourcel et al. created an online database based on its own MLVA scheme (http//:mlva.u-psud.fr) that allows users to create their own account to manage personal data. Different VNTR markers could be selected by the users to query the stored database, which may be seen as a repository of MLVA data (329).

Shouls et al. created a reference online database hosted at the Dutch National Institute for Public Health and the Environment (RIVM) and available at http://www.mlva.net. The database includes over 2550 different MLVA profiles and 23 MLVA complexes obtained from over 11000 Dutch *S. aureus* isolates and additional 2500 isolates from 23 other European countries. This database constitutes a MLVA typing tool that allows interrogation of MLVA types by the users.

2.5. Translating typing results into useful information for infection control

Microbial typing is widely applied, directly or indirectly, in clinical practice. Reports of typing results are important in outbreak situations and in long term surveillance, to decide on infection control measures (319). However, the application and interpretation of molecular typing in clinical and epidemiological contexts requires, not only the understanding of the typing results, but also the inclusion of epidemiological data concerning the patients (291, 319). The introduction of the concept of pathogen profile, which integrates phenotypic and genotypic typing results with additional clinical and epidemiological data, constitutes a valuable tool of communicable disease control (291). Therefore, the mutual contribution of the laboratory and the clinical practitioners is of major importance.

Moreover, the feedback following typing is essential and must include all those involved, not only to translate the results into practice, but also to guarantee continued motivation in maintaining the infection control measures and surveillance screenings (319).

3. Epidemiology of *S. aureus*: contemporary scenario

3.1. Evolution and global dissemination of MRSA clonal lineages

Two theories addressed the origins of the major contemporary MRSA clones: (i) the single-clone theory proposes that all MRSA were descendent from a single ancestral and that the SCC*mec* was introduced only once into this *S. aureus* strain (171); (ii) the more recent multi-clone theory suggests that SCC*mec* has been introduced several times into various *S. aureus* lineages (82, 106, 224).

In order to clarify the origins and spread of MRSA, Crisóstomo et al. studied a collection of MSSA and early MRSA strains isolated in the 1950/60s in Denmark and UK, where the first MRSA were identified and preserved (59). The authors noticed a gradual accumulation of resistance traits in MSSA paralleling the antimicrobial introduction in therapeutics, and suggested that these MSSA presenting a genetic background similar to the early and contemporary MRSA, represented the progeny of an *S. aureus* strain that must have been one of the first recipients of the *mec* element in the evolutionary history of MRSA (59). These findings were further confirmed by another study of Danish *S. aureus* isolates recovered in the 1960s from blood infections (106). The early MRSA, characterized by ST250 and SCC*mec* type I, was named “Archaic” clone (59, 245).

The characterization of more than 3,000 MRSA isolates from surveillance and outbreak studies in Southern and Eastern Europe, North and Latin America and Asia, by the CEM/NET initiative between 1994 and 2000 evidenced the spread of six major clones (310). Each clonal lineage was defined by a specific genetic background: the Iberian (ST247-IIA), Brazilian (ST239-IIIA), Hungarian (ST239-III), New York/ Japan (ST5-II), Pediatric (ST5-VI) and EMRSA-16 (ST36-II)

MRSA clones (3, 63). Two additional clones, the EMRSA-15 (ST22-IV) and Berlin (ST45-IV) showed epidemic potential, namely in the United Kingdom and Germany (149, 220, 336, 345).

Enright et al. characterized by MLST and SCC*mec* typing a wide collection of MRSA and MSSA isolated in 20 countries between 1961 and 1999, and defined that the major MRSA clones were associated to five clonal lineages: CC5, 8, 22, 30 and 45. Moreover, the study has shown that the successful ST8-MSSA lineage, in CC8 was the ancestor of the Archaic clone (ST250-MRSA-I), since ST250 differs from ST8 by a single point mutation in the *yqiL* locus (82). Evolutionary schemes were drawn for each of the five major CC, based on point mutations in housekeeping genes, recombination events and acquisition of SCC*mec* (Figure 3) (82, 106, 272).

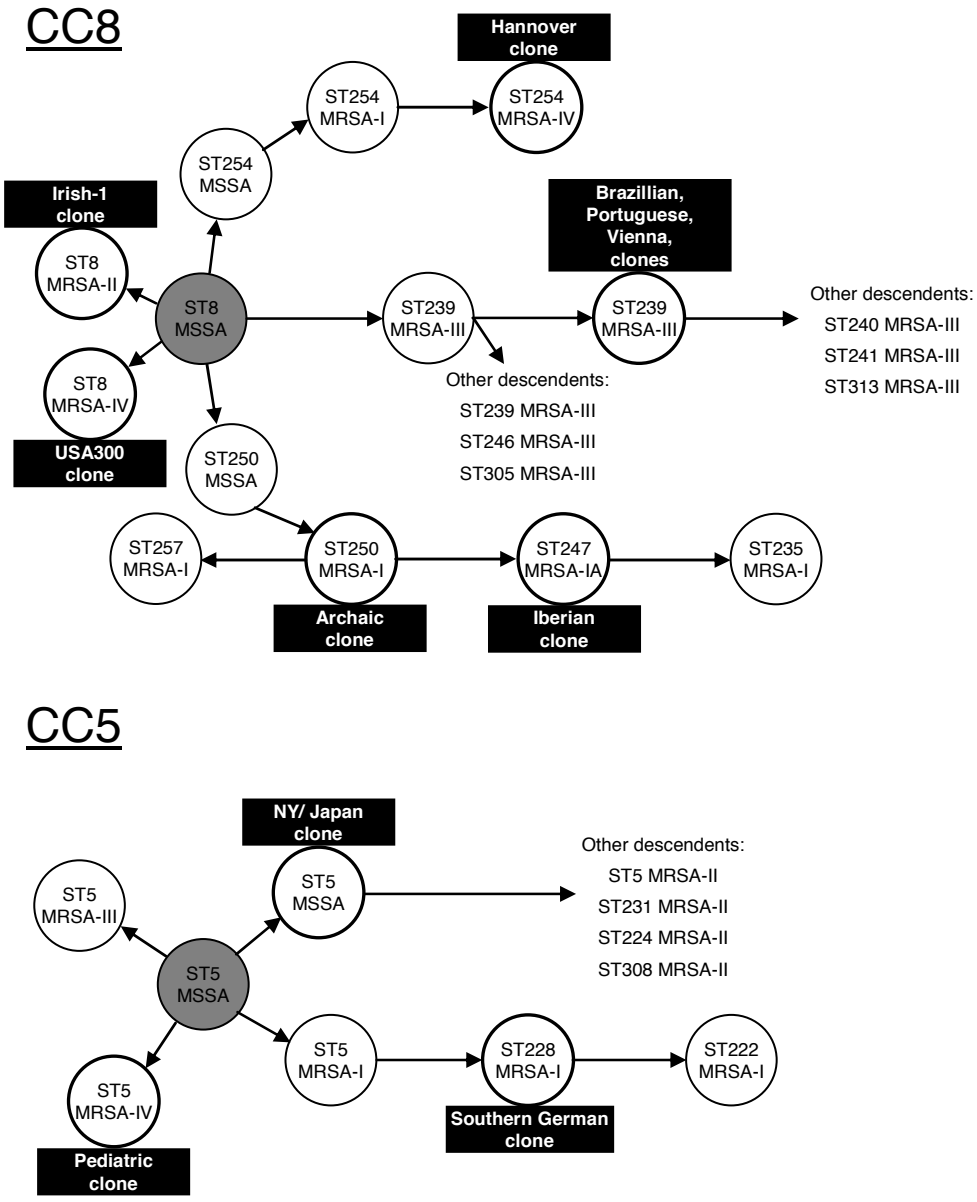

Figure 3. Evolutionary origins of the main MRSA clones belonging to each major clonal complex (CC), CC8, CC5, CC22, CC30 and CC45. Adapted from (82, 106, 272, 273).

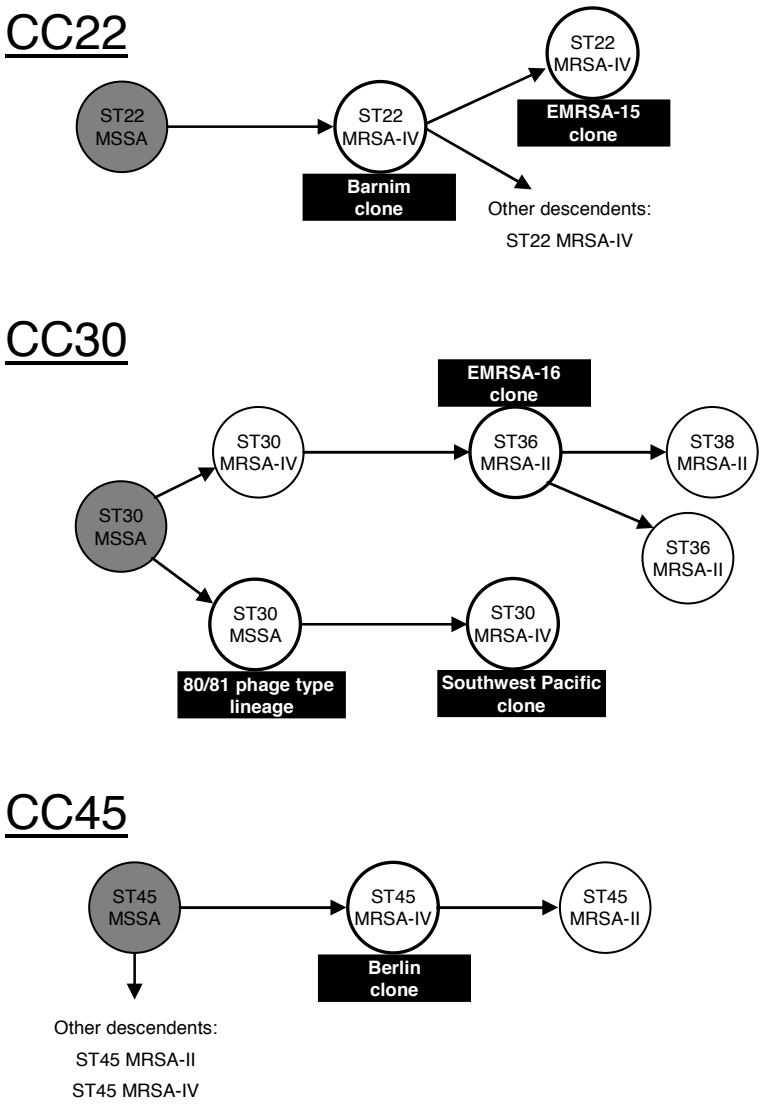

Figure 3. *Continued*.

In addition to ST250-MRSA-I, CC8 included the genetic background for other successful MRSA lineages such as the ST247-MRSA I (Iberian clone), the ST239-MRSA-III (Brazilian and Hungarian clone), ST254-MRSA-IV (Hannover clone), ST8-MRSA-II (Irish-1 clone) and one of the most virulent and widespread community acquired clones, the ST8-MRSA-IV PVL positive USA300 (82, 272,

305). ST239-MRSA-III is a pathogenically distinct and clinically important branch of CC8 that arose by the exchange of a ~557kb chromosomal fragment of the ST30 into the ST8 genetic background (271).

CC5 originated from an ancestral ST5-MSSA that diverged into major actual clonal lineages, as the ST5-MRSA-II (New York/ Japan clone), the ST5-MRSA-IV (Pediatric clone) and ST228-MRSA-I (Southern German clone). Moreover, the first VISA and VRSA isolates reported evolved from the ST5-MRSA-II genetic background (37, 132). A recent study on the CC5 evolution showed that variations of this successful lineage emerged by temporal and geographic independent SCC*mec* acquisitions (236).

In CC22 lineage, an ST22-MSSA was the predicted precursor of ST22-MRSA-IV known as the Barnim or EMRSA-15 clone, depending of the geographic origin of the isolates. In CC45, the ancestor ST45-MSSA precedes the ST45-MRSA-IV background of the Berlin clone.

In CC30, the ST30-MSSA was thought to be the ancestral of two currently distinct clonal types: the ST36-MRSA-II, the hospital acquired EMRSA-16, and the community acquired ST30-MRSA-IV PVL positive Southwest Pacific clone (272, 273).

SCC*mec* seems to be a driving force for evolution, hence MRSA has emerged at least 20 times upon SCC*mec* acquisition, which is four times more common than the replacement of one SCC*mec* cassette by another, and the smallest cassette type, type IV, was found in twice as many clones as any other SCC*mec* (272).

3.2. Methicillin-resistant *S. aureus* (MRSA) in the nosocomial setting

Defined risk factors for hospital acquired (HA-) MRSA infection include prolonged hospitalization or residence in a nursing home, patients undergoing dialysis, indwelling catheters, ICU exposure, prior antimicrobial therapy, surgical procedures, history of anterior MRSA infection or colonization, and close proximity to an inpatient colonized with MRSA (162). Moreover, the overwhelming majority of HA-MRSA infections are associated with invasive disease, namely pneumonia, blood stream, surgical wound and urinary tract infections (43). The growing impact

of antibiotic use in addition to more invasive medical interventions and comorbidities of the patients seems to add to the burden of MRSA infections (30).

HA-MRSA are usually multiresistant, harbor SCC*mec* cassette types I, II, III and IV, which include plasmids and transposons carrying additional antibiotics and heavy metals resistance genes (66, 92).

Several epidemiologic studies, including the SENTRY Antimicrobial Surveillance Program determined that between 1997 and 2003 the global MRSA prevalence in hospitals was 23% in Australia, 67% in Japan, 40% in South America, 36% in the United States and 23% in Europe (18, 67, 114, 308). Additional SENTRY data reveal the increasing trend in the proportion of MRSA in Latin America from 33.8% in 1997 to 40.2% in 2006 (256). Moreover, the Pan-American Association of Infectious Diseases reported for the same year of 2006 rates of HA-MRSA ranging from 25% in Ecuador to more than 50% in Argentina, Bolívia and Brazil (117).

In the Unites States' nosocomial setting, the proportion of HA-MRSA has been increasing over time, from 2% of the total *S. aureus* isolates in 1974, to 22% in 1995, 60% in 2003 and 64% in 2004 (160, 227). However, between 2005 and 2008, a population-based surveillance study reported a 9.4% and a 5.7% yearly decrease amongst hospital-onset invasive infections and heath-care associated community-onset infections, respectively. In the same study, a decrease of the hospital-onset infections (from 26% to 23%) was paralleled by an increase of the community-associated infections (from 17% to 19%) (152).

In Europe, the 2008 report from the European Antimicrobial Resistance Surveillance System (EARSS) stated that 21% of the invasive *S. aureus* were identified as MRSA and the geographic prevalence varied considerably, from less than 1% in the north to over 50% in southern nations. While several countries from Central and Eastern Europe have been showing a decreasing trend in MRSA occurrence, Malta and Portugal reported still rising MRSA proportions of 56% and 53%, respectively (83). Currently, a few highly epidemic MRSA clones are spread worldwide (Table 2).

Table 2. Molecular characterization and geographic distribution of the major HA-MRSA clones Adapted from (66).

HA-MRSA clone	MLST profile	ST	CC	SCC*mec*	*spa* types	Geographic spread
New York/Japan (USA100)	1-4-1-4-12-1-10	5	5	II	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Ast, Bel, Can, Chn, Den, Fin, Fra, Ger, Hun, Ire, Isr, Jap, Kor, Mex, Nor, Por, SA, Sin, Swe, Swi, Tai, Uru, UK, USA
Pediatric (USA800)	1-4-1-4-12-1-10	5	5	IV	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Alg, Arg, Ast, Bra, Col, Den, Fin, Fra, Kor, Nor, Pol, Por, Spa, Swe, Uru, UK, USA
Southern Germany	1-4-1-4-12-24-29	228	5	I	t001, t023, t041, t188, t201	Aus, Bel, Den, Fin, Ger, Hun, Ita, Slo, Spa, Swi
UK EMRSA-3	1-4-1-4-12-1-10	5	5	I	t001, t002, t003, t010, t045, t053, t062, t105, t178, t179, t187, t214, t311, t319, t389, t443	Arg, Den, Nor, Par, Pol, Por, Slo, Tai, UK
Archaic	3-3-1-1-4-4-16	250	8	I	t008, t009, t194	Ast, Can, Den, Ger, Swi, Uga, UK, USA
Brazilian/Hungarian	2-3-1-1-4-4-3	239	8	III	t030, t037, t234, t387, t388	Alg, Arg, Ast, Aus, Bra, Can, Chi, Chn, Cze, Den, Fin, Ger, Gre, Hun, Ind, Ids, Kor, Mon, Net, Nor, Par, Pol, Por, SA, Sin, Slo, Spa, Sri, Swe, Tai, Tha, UK, Uru, USA, Vie
Iberian	3-3-1-12-4-4-16	247	8	I	t008, t051, t052, t054, t200	Aus, Bel, Cro, Cze, Den, Fin, Fra, Ger, Hun, Isr, Ita, Net, Nor, Pol, Por, Slo, Spa, Swe, Swi, UK, USA
Irish-1	3-3-1-1-4-4-3	8	8	II	t008, t024, t064, t190, t206, t211	Ast, Can, Ire, UK, USA
UK EMRSA-2/-6 (USA500)	3-3-1-1-4-4-3	8	8	IV	t008, t024, t064, t190, t206, t211	Ast, Aus, Bel, Can, Den, Fin, Fra, Ger, Hun, Ire, Isr, Net, Nor, Swi, Tai, UK, USA
UK EMRSA-15	7-6-1-5-8-8-6	22	22	IV	t005, t022, t032, t223, t309, t310, t417, t420	Ast, Aus, Bel, Can, Chn, Cze, Den, Fin, Ger, Hun, Ire, Kuw, NZ, Nor, Por, Sin, Spa, Swe, UK
UK EMRSA-16 (USA200)	2-2-2-2-3-3-2	36	36	II	t018, t253, t418, t419	Ast, Aus, Bel, Can, Den, Fin, Gre, Ire, Mex, Nor, Por, Spa, Swe, Swi, UK, USA
Berlin (USA600)	10-14-8-6-10-3-2	45	45	IV	t004, t015, t026, t031, t038, t050, t065, t204, t230, t390	Arm, Ast, Aus, Bel, Chn, Den, Fin, Ger, Hun, Isr, Net, Nor, Spa, Swe, Swi, USA

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

3.3. Methicillin-resistant *S. aureus* (MRSA) in the community

The notion that MRSA infections are confined to the nosocomial setting changed in the early 1990s with the increasing number of MRSA infections in the community, reported in otherwise healthy persons without established health care risk factors for MRSA. These infections were apparently acquired in the community and have been referred to as community acquired or community associated MRSA (CA-MRSA) infections (60). The first truly cases of CA-MRSA infection were reported among Aboriginal patients in remote communities in Western Australia during 1993 (316). Afterwards, in 1997-1999, four otherwise healthy children died from sepsis and necrotizing pneumonia due to CA-MRSA in Minnesota and North Dakota (39). Since then, CA-MRSA was recognized as a new public health treat.

3.3.1. CA-MRSA definition and predisposing risks for infection

The Center for Disease Control and Prevention (CDC) defined CA-MRSA infection as “any MRSA infection diagnosed for an outpatient or within 48 h of hospital admission”. The patient’s medical history cannot contain MRSA infection or colonization, hospitalization or residence in a long-term care facility during the previous year, hemodialysis or surgery. Furthermore, the patient should not carry indwelling catheters or percutaneous medical devices (74, 221). However, the previous criteria were not always met and a clear delineation between CA-MRSA and HA-MRSA is not straightforward and the CDC proposed the definition of “health care associated, community-onset” MRSA for infections in patients with history of health care exposure but originated in the community (161). Additionally, recent reports of CA-MRSA infections in health care settings have been bluring the boundaries of CA-MRSA definition (160, 162).

Although skin and soft tissue infections (SSTI) such as furuncles, boils and abscesses are the most common clinical manifestations of CA-MRSA infections (39), some CA-MRSA have been associated to more invasive disease as folliculitis, cellulites, impetigo, pyomyositis and myositis, septic arthritis, osteomyelitis, sepsis, endocarditis, necrotizing pneumonia and necrotizing fasciitis (12, 205, 208, 249, 269).

Specific population groups are at higher risk of infection, namely children and young people (130), injecting and intranasal drug users (137), people of low socioeconomic status (42), athletic and competitive sport players (40), prison inmates and soldiers (41, 80), men who have sex with men (69), ethnically closed or semi-closed communities such as Pacific Islanders (38), rural American Indian and Southwester Alaska populations (112) and remote Aboriginal communities (316).

CA-MRSA is highly transmitted by direct contact, usually skin-to-skin or to contaminated fomites (40, 206), therefore the CDC proposed the six C's that promote CA-MRSA transmission: crowding, contact, cleanliness, compromised skin integrity, contaminated objects and exposure to antibiotic capsules (206).

3.3.2. CA-MRSA genetic markers

CA-MRSA isolates show some specific phenotypic and genotypic traits producing infections clinically and epidemiologically different from HA-MRSA. CA-MRSA strains are mainly susceptible to non-beta-lactam antibiotics and have a faster growth rate (92, 240). In addition, these isolates frequently produce the PVL leukotoxin, carry SCC*mec* types IV, V or VII, and the accessory gene regulator genotype I or III (66, 246, 300, 313). The association of CA-MRSA to smaller SCC*mec* cassettes seems to offer fitness advantage in the community setting (240).

PVL has been considered a genetic marker for CA-MRSA PVL-positive CA-MRSA isolates have been epidemiologically linked to recurrent and severe skin infections and necrotizing pneumonia (104, 180). However, reports of successful CA-MRSA PVL-negative clonal lineages have raised some controversy about the effective role of this leukotoxin gene as a specific marker for CA-MRSA (64, 238).

In fact, some studies suggested that other factors than PVL are enrolled in pathogenicity (68, 111). Sequencing of the successful CA-MRSA USA300 genome revealed the presence of the mobile genetic element ACME that is believed to confer fitness advantage and enhanced transmissibility (71). Moreover, recently, Wang et al. identified α-type phenol-soluble modulins (PSMα) that contribute

significantly to the CA-MRSA ability to cause disease, associated to the production of gama-hemolysin (335). Bubeck Wardenburg at al. also showed that α-toxin (or α-hemolysin), a pore forming toxin that lyses many types of host cells (including leukocytes, but not neutrophils) is also involved in CA-MRSA disease and pathogenesis (32).

3.3.3. Geographic distribution and clonal dynamics of CA-MRSA

Successful PVL-positive CA-MRSA clones were initially associated to a continent specific geographic location (328). The European clone, ST80-IV was spread in Europe (328) while in the United States predominated the USA300 clone (ST8-IV), in addition to USA400 (ST1-IV) and USA1000 (ST59-IV) (248, 270). The Southwest Pacific clone (ST30-IV) was found in Asia and Oceania, and the Queensland clone (ST93-IV) existed in Oceania only (133, 330). However, this picture is smearing since nowadays five major PVL-positive CA-MRSA clones have disseminated worldwide (Figure 4) (313).

While the European clone is currently found in Europe, North Africa, Singapore and in the Middle-East, USA300 became widely dispersed in the United States and Europe. In Japan, ST8-IV is the prevalent CA-MRSA clone, but is distinct from USA300 since it does not contain PVL or ACME. USA400, beyond the United States, is found in Europe, Australia and Asia. The Southwest Pacific clone is found in Australia, Europe, South America and South East Asia (China and Japan). USA1000 spread from the United States to the Asiatic continent, namely to Taiwan and China (60, 64, 313, 352).

Notwithstanding the major CA-MRSA clonal lineages, some PVL-positive minor clones emerged in more restricted geographic areas, despite the Queensland clone (ST93-IV) that migrated from Oceania to Europe and clone ST377-V currently disseminated in Australia, France, Greece, Switzerland and The Netherlands (Figure 4) (66). Moreover, in Taiwan the prevalent ST59 clone is usually found associated to two SCC*mec* cassettes: types IV and V, but only ST59-V is mostly PVL positive (300).

On the other hand, PVL-negative CA-MRSA are prevalent among the Australian ST1, the Japanese ST8-IV and the Korean ST72 clonal lineages, but

also found in minor clonal backgrounds as ST5 and ST129 in Australia and ST88 in Africa (Figure 4) (64, 159, 238, 301).

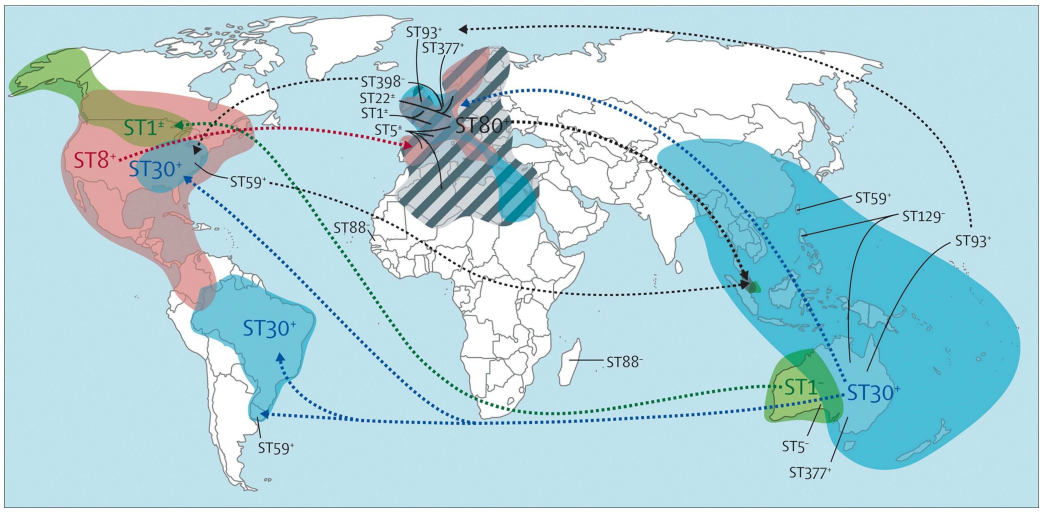

Figure 4. Global distribution and routes of dissemination of CA-MRSA lineages. Dotted lines indicate possible route of strains dissemination and color areas identify the extent of spread of major CA-MRSA clonal lineages infections, ST1 (green), ST8 (red), ST30 (blue), and ST80 (grey hatched). +, PVL-positive strains; –, PVL-negative strains; ±, combination of PVL-positive and PVL-negative strains. Reprinted with permission from (64).

In the Northern European countries, were the prevalence of HA-MRSA is very low, CA-MRSA accounts for the increasing incidence of MRSA infections in both the nosocomial and community settings (89, 176). The emergence of CA-MRSA infections among hospitalized patients with risk factors for MRSA infection suggests that the distinction between CA- and HA-MRSA is blurring (160, 162).

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

Until recently, MSSA was mostly regarded as the receptor of the SCC*mec* and predictive ancestor of epidemic MRSA lineages, which allied to the increased burden produced by the MRSA infections in nosocomial and community settings, relegated to a secondary plan the MSSA importance as a human pathogen.

Global assessment of *S. aureus* carriage rates in different populations showed that the overwhelming majority of the isolates (ranging from 97% to 100%) are MSSA (28, 46, 192, 302). Moreover, recent studies report the increasing number of invasive disease caused by MSSA, often resulting in a fatal outcome,

namely in children (110, 207, 219, 253, 275). Data also showed that MSSA can persist for long periods in the ICUs (107).

Community acquired MSSA (CA-MSSA) infections associated to SSTI have been also reported (218, 309). In two pediatric hospitals in the United States, the number of cases of CA-MSSA ranged from 25% to 45% of the total CA-*S. aureus* infections and CA-MSSA were more likely to be associated with invasive infections than CA-MRSA (211, 218).

Although methicillin resistance was associated to high mortality rates, increased length of hospitalization and hospital costs (54, 260), several studies showed an identical outcome for MSSA infections and therefore virulence was not correlated with resistance (207, 219, 311). In addition, to date, there is no compelling evidence that MRSA are more virulent than MSSA isolates (277).

The pathogenicity of MSSA strains is believed to be associated to the presence of a variety of virulence factors often also carried by MRSA (155). Of major interest is the increasing prevalence of MSSA isolates carrying PVL (217, 262). A recent study on the global distribution of MSSA isolates showed that most predominant genetic backgrounds of PVL-positive MSSA are pandemic and are phylogenetically related to CA-MRSA (262). In addition, infections caused by CA-MRSA or CA-MSSA present similar epidemiologic and clinical characteristics (130, 311) reinforcing the idea of MSSA as a genetic reservoir of MRSA.

Despite the wide genetic diversity among the MSSA population, some major lineages corresponding to the genetic background of the major MRSA clones were described worldwide, and in certain cases co-existed in the hospital and community (81, 113, 119). Some *S. aureus* lineages were found to be associated specifically to methicillin susceptibility, as the case of the successful ST121. ST121 frequently carries PVL and was already present in the early MSSA isolates from Denmark (106, 217). This MSSA lineage has been isolated in geographic distinct locations associated to both nasal carriage and disease, namely SSTI in children (15, 262). Of major concern was the recent report of methicillin resistance in three ST121 isolates in pediatric communities in China and Cambodia: one isolate, PVL negative responsible for staphylococcal scalded skin syndrome and two PVL positive, SCC*mec* type V isolates responsible for

osteomyelitis and soft tissue abscess (45, 85). Moreover, additional ST121 MRSA isolates were reported in the United States (248), which means that the *S. aureus* epidemiology is dynamic and under continuous evolution.

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Chapter II

Comparison of genetic backgrounds of methicillin-resistant and susceptible *Staphylococcus aureus* isolates from Portuguese hospitals and the community

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ABSTRACT

In order to understand the origins of the dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clones in Portuguese hospitals, we compared the genetic backgrounds of nosocomial MRSA with methicillin-susceptible *S. aureus* (MSSA) isolates from the same hospitals (*n* = 155) and from the community (*n* = 157) where they were located. Pulsed-field gel electrophoresis, *spa* typing, multilocus sequence typing, and *agr* type analysis revealed that the genetic backgrounds correspondent to the dominant MRSA clones in Portuguese hospitals during the last 15 years (Iberian ST247, Brazilian ST239, and EMRSA-15 ST22) were scarcely or not found among the present MSSA collection. The four major MSSA clones encountered (A-ST30, B-ST34, C-ST5, and H-ST45) correspond, or are very similar, to the background of other international MRSA pandemic clones, i.e., EMRSA-16, New York/Japan, Pediatric, and Berlin clones. However, with the exception of the Pediatric clone, none of these MRSA clones has been detected in Portugal. Our findings suggest the three major MRSA clones identified in Portuguese hospitals have not originated from the introduction of SCC*mec* into dominant MSSA backgrounds present in the Portuguese nosocomial or community environment but were probably imported from abroad. In contrast, the MRSA Pediatric clone might have originated in our country by the acquisition of SCC*mec* type IV into MSSA clone C. Furthermore, we provide evidence that the introduction of SCC*mec* into sensitive clones is most likely a relatively infrequent event that seems to depend not exclusively on the presence of a successful MSSA lineage.

INTRODUCTION

Staphylococcus aureus is a major human pathogen capable of causing a wide range of infections of different severity, such as skin abscesses and wound infections, osteomyelitis, endocarditis, pneumonia, meningitis, bacteremia, and toxic shock syndrome. Gradually, *S. aureus* has evolved resistance to all classes of antibiotics (35). Methicillin-resistant *S. aureus* (MRSA) strains have emerged by acquisition of a mobile genetic element called staphylococcal chromosomal cassette (SCC) *mec*, which carries the *mecA* gene. Five different types of SCC*mec* (types I to V), which differ in size and structure, have been described for *S. aureus* (11, 12, 17).

It is now clear that methicillin resistance has emerged by the introduction of SCC*mec* into at least five phylogenetically distinct successful methicillin-susceptible *S. aureus* (MSSA) lineages (5, 27) resulting in a relatively small number of pandemic MRSA clones spread worldwide, namely the Iberian (ST247-SCC*mec* IA), Brazilian (ST239-III), New York/ Japan (ST5-II), Pediatric (ST5-IV), EMRSA-16 (ST36-II), EMRSA-15 (ST22-IV), and Berlin (ST45-IV) clones (1, 7, 26). On the other hand, there is evidence that resistance has been transferred to *S. aureus* on more than five occasions, as some lineages have acquired different structural types of SCC*mec* (7, 27).

The nosocomial prevalence of MRSA in Portugal was estimated as close to 50% between 1993 and 1997 (19, 20) and 47.5% in 2003 (J. Melo-Cristino, personal communication). The European Antimicrobial Resistance Surveillance System reported an MRSA prevalence in Portugal in blood isolates of around 39% in 2002, which is one of the highest in Europe (32). During the last 15 years there were successive waves of dominant MRSA clones in Portuguese hospitals: (i) in 1992 and 1993, the Iberian clone replaced the Portuguese clone (ST239-III variant) widely spread in the country in the mid-1980s and early 1990s; (ii) in 1994 and 1995, the Brazilian clone was introduced, and its representation has rapidly increased since then; and (iii) in 2001, clone EMRSA-15 emerged and is nowadays becoming the dominant clone in the hospital setting (1). In addition, the

Pediatric clone was described for the first time in a pediatric hospital in Lisbon in coexistence with the Iberian clone between 1992 and 1996 (30).

Although reports of community-acquired MRSA (CA-MRSA) are increasing worldwide, the single study evaluating the prevalence of MRSA among the Portuguese community revealed a low carriage rate (<1%) (29). In that study, the two out of seven CA-MRSA isolates not health-care associated produced enterotoxins A and B and were both ST82.

In order to understand the origins of the nosocomial major MRSA clones present in Portuguese hospitals, we compared the genetic background of MRSA collected in different periods from three hospitals located in Lisbon and Oporto with MSSA isolates from those hospitals and from the community where they were located.

MATERIAL and METHODS

Bacterial strains

A total of 312 MSSA isolates divided into nosocomial (*n* = 155) and community (*n* = 157) collections were included in the present study.

Nosocomial isolates were collected from single patients at three Portuguese hospitals: Hospital Geral de Santo António (49 HSA isolates) and Instituto Português de Oncologia do Porto (39 IPOP isolates), both located in Oporto, and Hospital de São José (53 HSJ isolates) located in Lisbon. The three nosocomial subcollections included invasive and carriage isolates that were recovered in different periods corresponding to the successive waves of dominant MRSA clones in Portuguese hospitals, namely, 1992–1993 (introduction of the Iberian clone), 1995 (introduction of the Brazilian clone), and 2001 (emergence of clone EMRSA-15). HSA isolates were collected between December 1992 and February 1993, the HSJ isolates were collected between September and November 1995 (49 isolates), and one isolate in July 1996 and three isolates between January and February 1997 and the IPOP isolates were collected between January and December 2001. Also included in the nosocomial collection were 14 carriage isolates obtained between June and September 2003 from health care workers at Hospital São Francisco Xavier (Lisbon) (BOH isolates).

Community isolates were obtained from different subcollections. One collection originated from a community-based study on the prevalence of *S. aureus* colonizing healthy young Portuguese populations including children attending day care centers and draftees (29). From 1,331 children aged 0 to 5 years old attending 16 day care centers located in different geographical areas of Lisbon, nasopharyngeal samples were obtained between February and March 1996 and 1997. *S. aureus* was isolated from 210 children, from which 64 MSSA (DCC isolates) representing several day care centers were randomly selected. From 823 draftees, males and females aged 17 to 22 years old, nasal swab cultures were obtained between May and June 1996 and between October and November 1997. *S. aureus* was isolated from 280 draftees, out of which 77 were originally from Lisbon or Oporto and were all included in the present study

(draftees isolates). Finally, 16 isolates were obtained from nasal, pharyngeal, or nasopharyngeal swabs of children and adult patients who attended an outpatient clinic in Oporto, Instituto Nacional Ricardo Jorge (RJ isolates) (1992 and 1993).

DNA isolation, detection of *mecA* and PVL genes, and determination of *agr* type

Chromosomal DNA was extracted by incubating cells grown overnight in a solid medium in 20 μl of TE 1x (10 mM Tris, 1 mM EDTA, pH 8) with lysostaphin at 0.5 mg/ml for the lysis step for 30 min, followed by a denaturation step of 15 min at 95℃. The mixture was harvested at 13,000 rpm for 5 min, and 2 μl of the supernatant was used as DNA template in the PCRs. The presence of the *mecA* and Panton-Valentine leukocidin (PVL) genes was determined by PCR as described previously (16, 22). The accessory gene regulator (*agr*) group was determined by a multiplex PCR according to the methods of Jarraud et al. (13).

Molecular typing

Pulsed-field gel electrophoresis (PFGE) was performed as described by Chung et al. (3) on all 312 isolates. The resulting band patterns were analyzed by visual inspection followed by the analysis with the Bionumerics software (version 4.0; Applied Maths, Gent, Belgium) for relatedness evaluation. Dendrograms were generated from similarity matrixes calculated with the Jaccard coefficient, and patterns were clustered by unweighted pair group method with averages using an optimization of 0.25% and a tolerance of 1.0%.

Nosocomial IPOP and HSA isolates, community DCC isolates, and all remaining isolates that presented a PFGE pattern not observed among the previous collections were further characterized by *spa* typing (14, 31) (total, 171 isolates). As done previously (14, 31), *spa* types with similar repeat profiles were grouped together as part of a same lineage (*spa* lineage), which was identified in the present study by capital letters. Multilocus sequence typing (MLST) was performed in representatives of each PFGE type/*spa* lineage as described previously (6), with the exception that primer *arcC*F2 (5'-CCT TTA TTT GAT TCA CCA GCG-3') (4) was used. For *spa* typing and MLST, PCR products were

purified with a Wizard PCR Preps purification system (Promega, Madison, WI) and used as templates for sequencing of both strands at Macrogen, Seoul, South Korea. MLST alleles and sequence types (STs) were identified using the MLST database (http://www.mlst.net) hosted by Imperial College.

RESULTS

Genetic diversity

A total of 20 different PFGE patterns were found among the 312 MSSA isolates. Further characterization by *spa* typing distributed the isolates into 72 *spa* types. Isolates showing related *spa* types could be grouped in 19 *spa* lineages showing congruence between clustering by PFGE and *spa* typing, with the exception of *spa* lineages B and P, which were associated with two PFGE patterns each, B and Q and P and L, respectively. PFGE analysis versus *spa* typing analysis is depicted in Figure 1. MLST performed on representatives of each PFGE pattern and/or *spa* lineage identified 20 STs, confirming the existence of 20 distinct clones among the 312 MSSA isolates. Application of the eBURST algorithm to the 20 STs recognized 17 groups, which were defined as clusters of closely related STs in which a single difference in the allelic profile was tolerated and therefore considered to belong to 17 clonal complexes (CCs) (8). Figure 2 illustrates the molecular characterization of strains representing the 20 clonal types defined by PFGE, *spa* typing, and MLST as well as the CC assignment generated by eBURST. Interestingly, some clones belonging to different CCs presented a higher PFGE similarity than clones included in the same CC. For instance, clone PFGE L-ST188 showed a PFGE similarity of 63.2% with clone PFGE Y-ST106 but only 55.3% with clone PFGE M-ST573 (Figure 2). These discrepancies may be due to the different spectra of changes detected by the two typing methods. PFGE examines both nucleotide changes that are in specific restriction sites and changes that involve large insertions or deletions of DNA in the whole genome, while MLST detects nucleotide changes only within the seven amplified housekeeping gene fragments. Moreover, to complicate the interpretation, bands of the same size are assumed to be identical in PFGE and unrelated fragments that are indistinguishable by size can occur by chance, especially as the genetic distance between strains increases. Despite the fact that in one case in the present study the PFGE and allelic profile similarities were not connected, it did not constitute an issue for typing, since the isolates were sufficiently unrelated and classified into different clones.

Despite the high genetic variability, over half (61%) of the isolates belonged to four major clonal types (represented by more than 10% of the isolates) and three CCs: clone A (PFGE type A, *spa* type 33 or related, ST30, CC30), clone B (PFGE type B, *spa* type 497 or related, ST34, CC30), clone C (PFGE type C, *spa* type 2 or related, ST5, CC5), and clone H (PFGE type H, *spa* type 136 or related, ST45, CC45).

Hospital versus community MSSA isolates

Sixteen out of 20 clones, including the four major clones, A, B, C, and H, were present in both nosocomial and community settings (Figure 1). However, some of the major clones were clearly represented by different proportions: clone C was overrepresented in the hospital setting (chi homogeneous square test, $p = 0.039$), and clone B contained proportionally more isolates from the community (chi homogeneous square test, $p < 0.0001$).

Among the 155 nosocomial isolates, four major clones could be identified, clones A ($n = 34$, 22%), C ($n = 22$, 14%), and H and K ($n = 17$ each, 11%), whereas clone B ($n = 49$, 32%) followed by clone A ($n = 37$, 24%) embraced 86 out of the 157 (55%) community isolates (Figure 3). Clone K was characterized by PFGE type K, *spa* type 21 or related, and ST15. The clonal distribution among the nosocomial isolates varied according to the hospital: clone A was predominant in both hospitals in Oporto, while clones C and K were the principal clones in hospital HSJ located in Lisbon. A relatively large number of genetic backgrounds ($n = 7$) was found among the 14 isolates recovered from health care workers from another hospital in Lisbon, and the major clones included only three isolates each.

Although the community isolates belonged to two major clones, the different populations were not evenly distributed: children attending day care centers more often carried strains from clone B ($n = 32$, 50%) in comparison with draftees ($n = 14$, 18%) (chi homogeneous square test, $p = 0.001$). No significant difference was found in the clonal distribution of community isolates from Oporto versus Lisbon (data not shown). The isolates recovered from patients attending the outpatient clinic in Oporto (RJ isolates) were distributed into several clonal types, as happened with the nosocomial BOH isolates, rather than showing a

significant major clone, probably due to the reduced number of isolates representing these subcollections.

A considerable number of minor clones, each harboring a maximum of 5% of all isolates, was found among both the hospital and community isolates. Minor clones reached 30 and 21% of all hospital and community isolates, respectively (Figure 3).

MSSA versus MRSA background

Comparing the genetic backgrounds of the major MSSA clones (A, B, C, and H) with international pandemic MRSA lineages, including the main MRSA clones present in Portugal during the last 15 years, revealed that clones A and B correspond to slight variations (MLST single and double locus variants, respectively) of the genetic background of MRSA clone EMRSA-16 (ST36, SCC*mec* type II), clone C corresponds to the background of the New York/Japan (ST5-II) and Pediatric (ST5-IV) clones, and clone H corresponds to the background of the Berlin clone (ST45-IV). In addition, two minor MSSA clones, J (6% of all isolates) and T (one isolate), are related to two additional widely spread MRSA clones. Clone J (PFGE J, *spa* type 1 or related, ST8) corresponds to a single and a double locus variant of the Brazilian (ST239-III) and Iberian (ST247-IA) clones, respectively, and clone T corresponds to clone EMRSA-15 (ST22-IV). On the other hand, eBURST analysis showed that 67% of all isolates belonged to one of the five major CCs described for MRSA, i.e., CC30, CC5, CC45, CC8, and CC22 (Figure 1, right panel).

A dendrogram representing the clustering of the 20 MSSA PFGE types together with representatives of the seven major international MRSA clones (Iberian, Brazilian, New York/ Japan, Pediatric, EMRSA-15, EMRSA-16, and Berlin) confirmed the relatedness between MSSA found in the present study and MRSA pandemic clones (Figure 2).

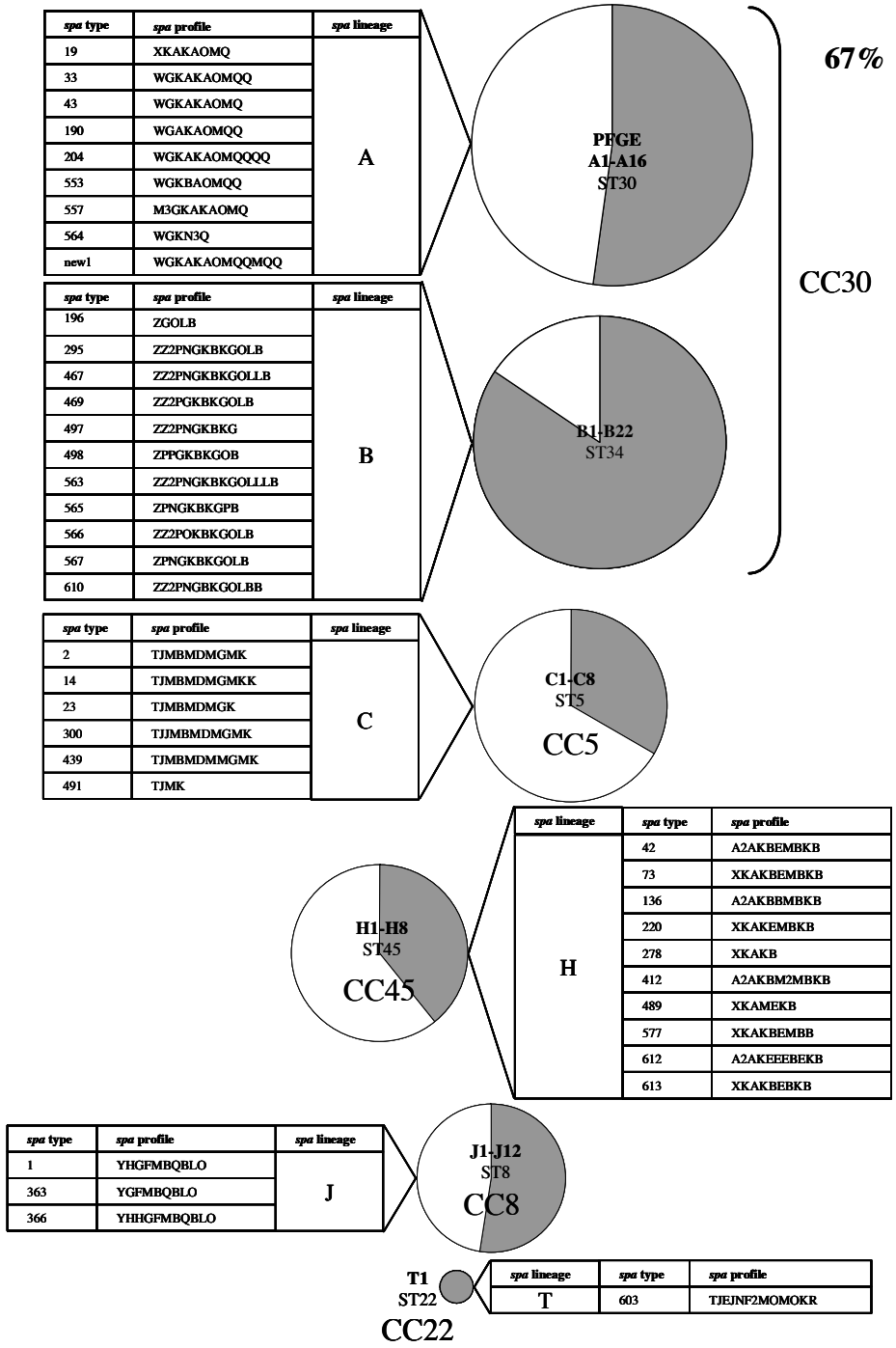


Figure 1. PFGE analysis versus *spa* type analysis of MSSA strains. Distribution of the 312 MSSA isolates into 20 PFGE types is shown. The area of each circle is proportional to the number of isolates included in the PFGE type, and white and gray areas correspond to nosocomial and community isolates, respectively. Subtypes are indicated for each PFGE type as well as the corresponding ST. *spa* type data, including *spa* type, *spa* profile, and *spa* lineage, are shown for 171 MSSA strains. PFGE types A, B, C, J, H, and T (left side of the figure) correspond to 67% of the total MSSA isolates and belong to the five major MRSA CCs

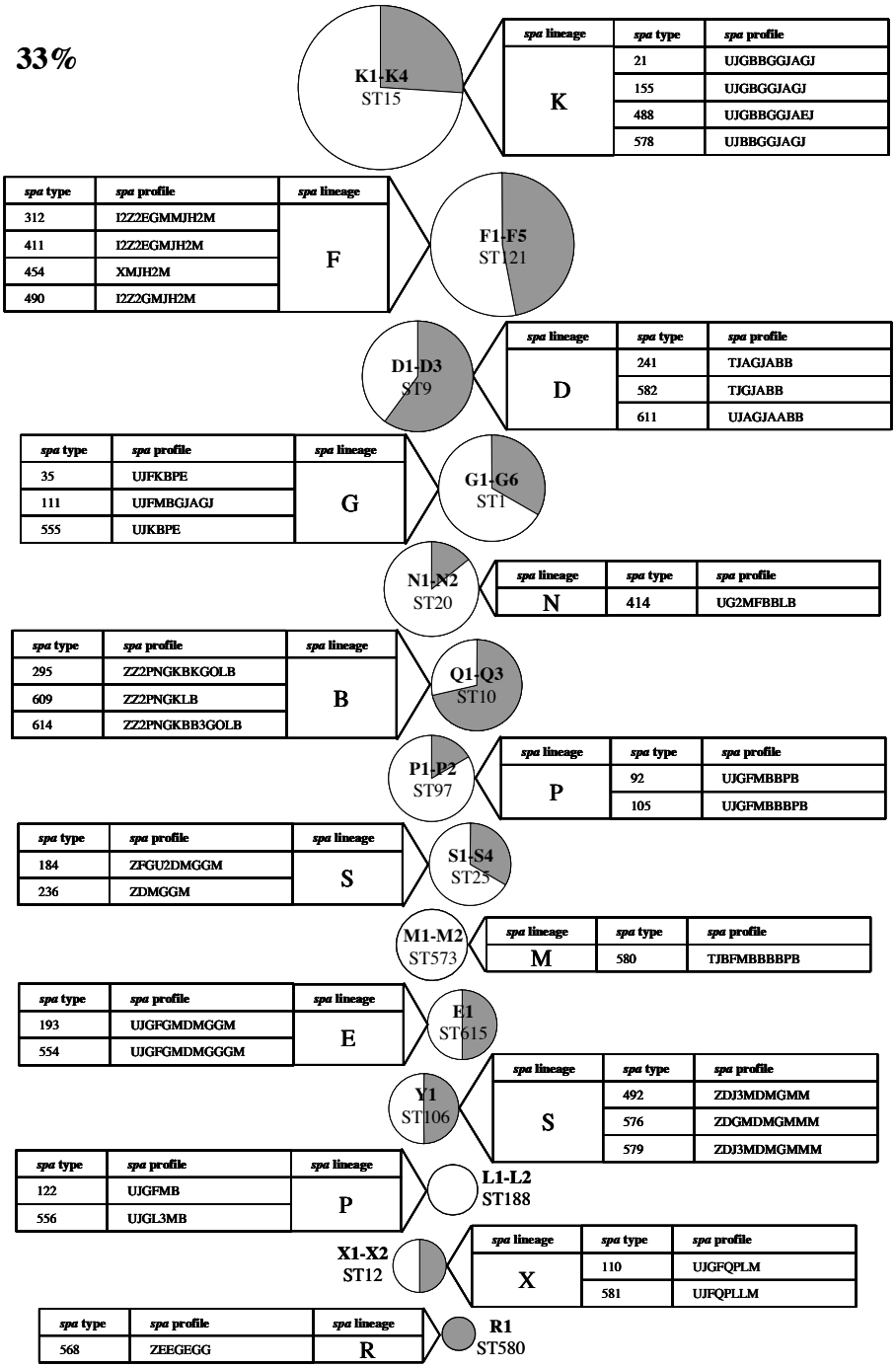
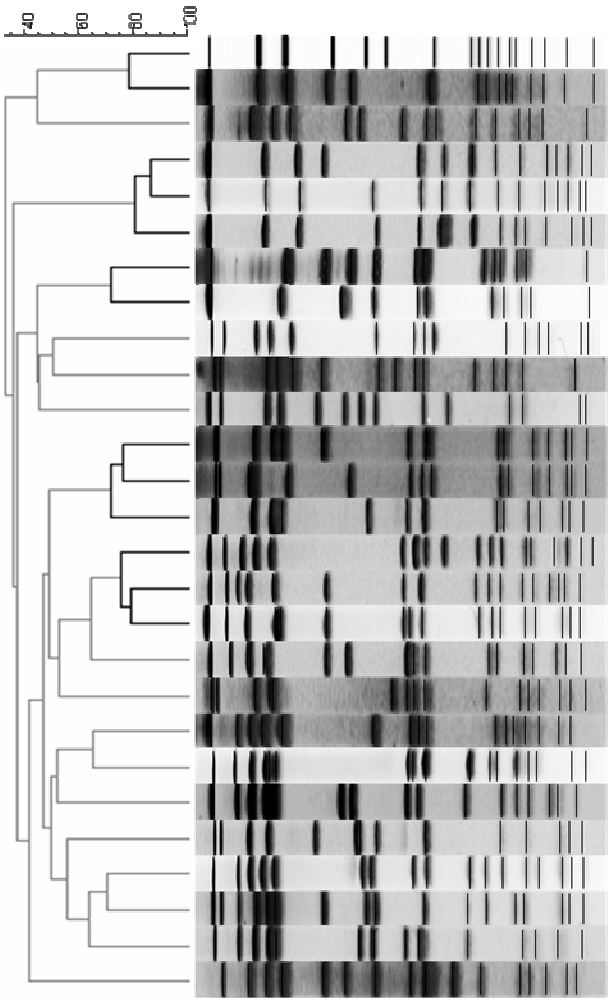


Figure 1. *Continued.*



Isolate	PFGE type	*spa* lineage	*spa* profile	*spa* type	*agr* type	ST	CC
825/96	**Berlin**	H	A2AKEEMBKB	15	1	45	45
DCC457	H	H	A2AKBBMBKB	136	1	45	
DCC750	S	S	ZFGU2DMGGM	184	1	25	25
96/32010	**EMRSA-16**	A	WGKAKAOMQQQ	16	3	36	30
DCC1060	A	A	WGKAKAOMQQ	33	3	30	
IPOP34	B	B	ZZ2PNGKBKG	497	3	34	
90/10685	**EMRSA-15**	T	TJEJNF2MNF2MOMOKR	436	1	22	22
DRAFTEES721	T	T	TJEJNF2MOMOKR	603	1	22	
IPOP33	D	D	TJGJABB	582	2	9	9
DCC1185	R	R	ZEEGEGG	568	1	580	?
IPOP74	F	F	I2Z2EGMJH2M	411	2	121	121
BK2464	**NY/ Japan**	C	TJMBMDMGMK	2	2	5	5
HDE288	Pediatric	C	TJMBDMGMK	45	2	5	
HSA29	C	C	TJMBMDMGMK	2	2	5	
HU25	**Brazilian**	A	XKAOMQ	390	1	239	8
HPV107	**Iberian**	J	YHFGFMBQBLO	4	1	247	
IPOP68	J	J	YGFMBQBLO	363	1	8	
HSA56	N	N	UG2MFBBLB	414	1	20	?
DCC192	K	K	UJGBBGGJAGJ	21	2	15	15
DCC403	E	E	UJGFGMDMGGM	193	1	615	72
DRAFTEES728	X	X	UJGFQPLM	110	2	12	12
IPOP50	P	P	UJGFMBBPB	92	1	97	97
HSJ109	M	M	TJBFMBBBBPB	580	2	573	1
IPOP38	G	G	UJFKBPE	35	3	1	
IPOP58	L	P	UJGFMB	122	1	188	
HSJ104	Y	Y	ZDJ3MDMGMMM	579	1	106	?
DCC300	Q	Q	XMJH2M	454	2	10	?

Figure 2. Molecular characterization of MSSA strains and comparison with MRSA pandemic clones. Shown from left to right are (i) a dendrogram indicating the estimated relationships of PFGE types based on Bionumerics analysis, including representatives of seven international pandemic MRSA clones (in bold), and squares filled with the same color or pattern indicate MSSA and MRSA backgrounds showing a relatedness of over 70%; (ii) listing of isolates; (iii) PFGE type; (iv) *spa* lineage, profile, and type; (v) *agr* type; (vi) MLST ST and CC. Œ, CC for which no founder could be assigned.

mecA and PVL gene amplification and *agr* specificity group

The presence of the *mecA* gene was determined by PCR amplification, and only *mecA*-negative isolates were included in the present study. We have assessed the prevalence of the PVL gene in isolates representing all clonal types from both the community and hospital populations. Two of 86 isolates tested were PVL-positive MSSA strains. These two isolates belonged to clone F (PFGE F, *spa* type 441, ST121). Interestingly, among the 17 strains belonging to clone F (9 from the hospital and 8 from the community), only 2 isolates harbored the PVL gene. These two isolates were found in the community among carriage isolates from a child attending a day care center and an Air Force draftee.

The determination of the *agr* specificity group for representatives of the 20 MSSA clones revealed that half belonged to *agr* type 1. The remaining clones belonged to *agr* type 2 (seven clones) and type 3 (three clones) (Figure 2). Although *agr* type 3 included three clonal types only, it was associated with the major clones A and B as well as clone G and therefore har-bored 44% of all MSSA isolates. Interestingly, the three clonal types belonging to CC1 showed three different *agr* types (Figure 2).

		Clonal types (%)						
		A-ST30	B-ST34	C-ST5	H-ST45	J-ST8	K-ST15	Other
	Nosocomial (n=155)	**34 (22)**	**9 (6)**	**22 (14)**	**17 (11)**	**9 (6)**	**17 (11)**	**47 (30)**
Oporto	Hospital HSA (n=49) 1992-1993	17 (36)	4 (8)	6 (12)	5 (10)	1 (2)	6 (12)	10 (20)
Lisbon	Hospital HSJ (n=53) 1995	6 (11)	0 (0)	10 (19)	4 (8)	4 (8)	9 (17)	20 (37)
Oporto	Hospital IPOP (n=39) 2001	8 (21)	3 (8)	6 (15)	5 (13)	4 (10)	0 (0)	13 (33)
Lisbon	Hospital BOH (n=14) 2003	3 (21)	2 (14)	0 (0)	3 (21)	0 (0)	2 (12)	4 (30)
	Community (n=157)	**37 (24)**	**49 (31)**	**11 (7)**	**11 (7)**	**10 (6)**	**6 (4)**	**33 (21)**
Lisbon	DCC (n=64) 1996-1997	11 (17)	32 (50)	4 (6)	5 (8)	1 (2)	3 (5)	8 (12)
Lisbon/ Oporto	Draftees (n=77) 1996-1997	22 (29)	14 (18)	6 (8)	6 (8)	7 (9)	3 (4)	19 (25)
Oporto	RJ (n=16) 1992-1993	4 (25)	3 (19)	1 (6)	0 (0)	2 (13)	0 (0)	6 (37)
	Total (n=312)	**71 (23)**	**58 (18)**	**33 (11)**	**28 (9)**	**19 (6)**	**23 (7)**	**80 (26)**

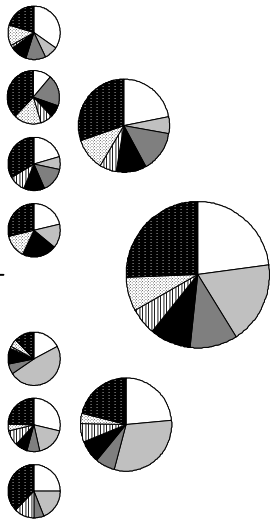


Figure 3. Clonal type distribution. Numbers underlined represent the major clonal types in each collection or subcollection. Minor clones (clones harboring a maximum of 5% of all isolates) were classified as "other" clonal types and include STs 1, 9, 10, 12, 20, 22, 25, 97, 106, 121, 188, 573, 580, and 615

DISCUSSION

The incidence of MRSA in Portuguese hospitals is one of the highest in Europe. We previously drew a temporal scheme for the evolution of MRSA clonal types in Portuguese hospitals (1). In the present study, the population structure of Portuguese MSSA, isolated from clinical and nonclinical isolates, has been determined. Among the nosocomial isolates four subcollections were used, corresponding to different hospitals and periods. The community subcollections were obtained from the geographical area served by the hospitals from children attending day care centers (<5 years) and Air Force draftees (17 to 22 years) with nasal carriage of *S. aureus* and from a small group of patients attending an outpatient clinic.

PFGE, *spa* typing, and MLST analysis distributed the 312 isolates into 20 clonal types. A search in the MLST database (http://www.mlst.net/) revealed that 15 out of the 20 STs identified in the present study are exclusively or mostly associated with MSSA isolates, evidencing a high genetic diversity among the MSSA population, as found by others (2, 6). Despite the high diversity, eBURST, a computer algorithm used to solve population structures based on MLST data (8), grouped 67% of the MSSA isolates, including clinical and carriage isolates, into four major clonal complexes (CC30, CC5, CC45, and CC8) corresponding to four of the five major MRSA clusters spread worldwide (7, 27, 28, 33). On the other hand, the main STs found in the present study have been found in other countries showing MSSA geographical spread. These observations support the evidence that the same main MSSA lineages seem to be disseminated in different regions of the world and that pandemic MRSA originated by the introduction of SCC*mec* into these most successful lineages. The fact that clinical and carriage strains as well as MSSA and MRSA isolates fall into the same main clusters is in agreement with Melles et al., who proposed that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen (18).

In comparison with the scenario observed for nosocomial Portuguese MRSA clones, the representation of the major MSSA clones in Portuguese hospitals appears to be more stable over time. Whether the slight differences in the clonal distribution between hospitals are due to the different sampling

moments or to the different geographic location of the hospitals is currently unclear. Comparison of the genetic backgrounds of the MSSA clones found in the present study with the backgrounds of the major international pandemic MRSA lineages revealed some overlapping. The major MSSA clones, A-ST30, B-ST34, C-ST5, and H-ST45, correspond or show a high degree of similarity in PFGE, *spa* type, and MLST to EMRSA-16 (ST36), the New York/ Japan or Pediatric clones (ST5), and the Berlin clone (ST45).

However, to the best of our knowledge, none of these clones with the exception of the Pediatric MRSA has been reported among nosocomial Portuguese MRSA isolates recovered between 1990 and 2001. The Pediatric MRSA clone was first described in a pediatric Portuguese hospital (30) and could have originated in our country by the introduction of a variant of SCC*mec* type IV (25) into MSSA clone C. We cannot rule out the hypothesis that the remaining main MSSA clones will not become major MRSA clones in Portugal in the future.

Interestingly, the genetic backgrounds corresponding to the three dominant MRSA clones in Portuguese hospitals during the last 15 years (Iberian-ST247, Brazilian-ST239, and EMRSA-15-ST22) or their ancestral genotypes were not detected or scarcely found among the present MSSA collection, clones J-ST8 (ancestral of ST239 and ST247) and T-ST22. Moreover, in the present collection the prevalence of the MSSA clone J increased between 1992 and 2001, which may indicate that most MSSA isolates belonging to this clone were probably isolated after the introduction of the Iberian clone (1992 to 1993) in Portuguese hospitals. These observations suggest the Iberian, Brazilian, and EMRSA-15 MRSA clones have not originated from the introduction of SCC*mec* into dominant MSSA backgrounds present in the Portuguese nosocomial or community environment but were probably imported from abroad.

The fact that there is no congruence between the genetic backgrounds of the major MSSA and MRSA clones within a population indicates that the presence of a successful MSSA lineage is not the sole factor necessary for SCC*mec* acquisition or for the success of an MRSA clone. Moreover, the introduction of SCC*mec* into sensitive clones seems to be a relatively infrequent event compared to the geographical spread of MRSA clones.

CA-MRSA isolates are reported to differ genetically from hospital-acquired MRSA (23). In contrast, community-acquired MSSA isolates are no different than hospital-acquired MSSA, as reported by Enright et al. (6). In the present study, clone A was overrepresented in the hospital setting, whereas clone B was the main clone in the community collection. Nevertheless, clone B was only predominant among isolates collected from children attending day care centers (50%). The different age categories may be an explanation for the observed difference. Moreover, confined environments such as day care centers, where children have close contacts, facilitate the easy spread of bacteria and therefore a high clonal expansion (15). Nevertheless, we cannot exclude the hypothesis that the restricted number of isolates is not a confounding factor.

PFGE and MLST analysis indicated distinct genetic backgrounds for CA-MRSA associated with each geographic origin, namely ST80 in Europe, STs 1, 8, and 59 in the United States, and ST30 in Oceania (33). Although in the present study we have not detected any MSSA sharing the background of the major European CA-MRSA clone, ST80, we detected MSSA isolates of STs 1, 8, and 30. ST1 and ST8 represented minor clones, but ST30, which was initially described among CA-MRSA in Australia and recently reported to have spread in the community in Europe both in The Netherlands and in Latvia (21, 34), corresponded to the main clone found in the present study. In addition, the background of the only two true CA-MRSA strains described so far in Portugal (29) was not found among the MSSA nasal carriage population in our work that in part overlaps the *S. aureus* isolates from the Sa-Leao et al. study (29). Apparently, as happened with the major hospital-acquired MRSA, we anticipate CA-MRSA ST82 was probably imported from abroad.

PVL is a toxin associated with *S. aureus* strains causing severe skin infections (16) and with highly virulent necrotizing pneumonia in young patients (9). We assessed the presence of PVL genes in representatives of each MSSA clonal type. The two single PVL-positive isolates belonged to clone F and were recovered in the community from one child and one draftee. The reason why only 2 out of the 17 clone F isolates harbored PVL may be due to the fact that PVL

determinants are carried by different temperate phages (24) and therefore should have integrated independently in the two isolates with background F.

As a further contribution to the genotypic characterization of the MSSA isolates, we determined the *agr* sequence types for representatives of each MSSA clone. The four successive major MRSA clones present in Portuguese hospitals during the last 15 years belonged to the same *agr* group 1 (10). However, the major MSSA and MRSA clones coexisting in the same hospital do not belong to the same *agr* type. It would be interesting to clarify the *agr*-associated interference between MSSA and MRSA. In addition, the fact that the three clonal types belonging to CC1 showed different *agr* types is intriguing and needs further investigation, since previous studies have linked the genetic background of *S. aureus* isolates to the *agr* type (10, 13).

In summary, the major hospital-acquired MRSA clones as well as the rare cases of CA-MRSA in Portugal are not related to the MSSA circulating in the nosocomial or the community setting in the country, which provides supportive evidence that SCC*mec* moves rarely and that pandemic MRSA spreads independently of the local MSSA clones.

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T. Conceição performed the experimental work concerning the community isolates (DCC, Draftees and RJ isolates), analyzed the global results and assisted in the design of the manuscript's figures.

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Chapter III

High prevalence of ST121 in community associated methicillin-susceptible *Staphylococcus aureus* lineages responsible for skin and soft tissue infections in Portuguese children

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ABSTRACT

In order to evaluate the incidence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Portugal, we analyzed a collection of 38 *S. aureus* isolates recovered from 30 children attending the pediatric emergency department of a central hospital in Lisbon due to skin and soft tissue infections. Molecular characterization identified seven clonal lineages among the 35 methicillin-susceptible *S. aureus* (MSSA) isolates, of which the major lineage PFGE A/t159/ST121 included 63% of the isolates. The three MRSA isolates belonged to the Pediatric clone PFGE D/t535/ ST5-IV (*n* = 2) and to the European CA-MRSA clone PFGE G/ t044/ ST80-IVc. All isolates harbored several virulence factors, namely, leukocidins. Panton-Valentine leukocidin (PVL) was produced by isolates from five MSSA lineages and by the ST80 MRSA. Of interest, this is the first reported isolation of CA-MRSA ST80 in Portugal.

RESULTS and DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the community have been increasing worldwide and are mainly reported as skin and soft tissue infections (SSTI) in otherwise healthy young individuals (18, 29, 32). The Centers for Disease Control and Prevention (CDC) consider a community-associated MRSA (CA-MRSA) infection when the patient has no previous history of MRSA infection or colonization, surgery, dialysis, hospitalization, residence in a long-term care facility within the year before infection, presence of a percutaneous device or indwelling catheter or hospitalization >48 h before MRSA cultures (6, 13).

CA-MRSA isolates differ phenotypically and genotypically from hospital-associated (HA-) MRSA, namely, in its non-multiresistant antibiotic patterns, enhanced virulent gene content, including the acquisition of the necrotizing Panton-Valentine leukocidin (PVL) genes or the expression of α-type phenol-soluble modulins (PSMs) (18, 29). The staphylococcal cassette chromosome *mec* (SCC*mec*) type and the accessory gene regulator (*agr*) alleles are also differentially distributed: SCC*mec* types IV or V and *agr* type III are more commonly present in CA-MRSA, while SCC*mec* types I-III and *agr* type II are more typical of HA-MRSA (29). In contrast, community-acquired methicillin-susceptible *S. aureus* (CA-MSSA) isolates, responsible for a significant number of mild SSTI, do not differ from HA-MSSA (4, 28).

Although reports of CA-MRSA prevalence are increasing worldwide, there is no description of the actual scenario in Portugal, a country with >50% HA-MRSA infection, currently the highest in Europe (14). Previous studies dated from 1996 to 2009, including isolates from nasal swabs of young healthy individuals and nasopharyngeal swabs of children attending day care centers, reported an MRSA prevalence lower than 0.25% in the Portuguese healthy community (0.24% in 1996-1998 and 0.13% in 2006-2009) (4, 34, 37).

It is conceivable that the incidence of CA-MRSA in Portugal is underestimated partially because skin infection samples are not routinely cultured.

The aim of the present study was to assess the prevalence and molecular characterization of *S. aureus* in children attending the pediatric emergency department of a central hospital due to SSTI.

The pediatric emergency department of Hospital Fernando Fonseca, a large tertiary-care hospital (670 beds), is the second largest pediatric urgency unit in the urban area of Lisbon, Portugal, and receives approximately 180 children per day. Between August 2005 and October 2006, all children attending this unit due to SSTI were enrolled in the study. Samples were recovered by swabbing the largest area of skin infection or wounds with spontaneous or surgical drainage, or in case of severe SSTI from hemoculture. Whenever possible, nasal swabs were also performed. A questionnaire was filled in order to collect data on basic socio-demographic patient information, risk factors associated with skin infection, and description of the infection.

S. aureus isolates were first identified by conventional coagulase and catalase tests. Additional identification and susceptibility testing with a panel of 10 antibiotics (Figure 1) were performed by the semi-automatic VITEK2 system (bioMérieux, SA, France) and the disk diffusion method with D-test for clindamycin (9). All isolates were tested by polymerase chain reaction (PCR) for the presence of *mecA* (30) and also characterized by pulsed-field gel electrophoresis (PFGE) (8). The resulting SmaI patterns were analyzed by both visual inspection and automatically with BioNumerics software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium) (16, 23). Characterization by *spa* typing, multilocus sequence typing (MLST), and the *agr* allele type were performed on representative isolates of each PFGE type (1, 10, 20). The SCC*mec* was typed for all MRSA isolates (24, 25). Specific staphylococcal virulence determinants, including leukocidins, hemolysins, and super-antigenic toxins of each isolate, were determined by PCR (27, 38) (Figure 2). Categorical variables were compared using the χ^2 or Fisher's exact test when appropriated, considering p values of ≤0.05 being statistically significant, and odds ratio (OD) estimates using the SPSS software package version 11.5 (SPSS Inc., Chicago, IL, USA).

During the 15-month study period, 30 (73.2%) out of the 41 children who attended the emergency department due to SSTI were infected by *S. aureus*. The major clinical infection presentations were cutaneous abscesses (*n* = 12, 40%) and cellulitis (*n* = 10, 33.33%). A total of 38 isolates were recovered, of which three (7.9%) were MRSA. Although eight clonal types (Table 1) were identified among the isolates, 63% (*n* = 19) belonged to a single type: PFGE A, *spa* type t159, sequence type (ST) 121, *agr* type IV. The ST121 lineage was also the predominant lineage found in MSSA isolates through Europe and Russia (5, 33, 39) and an identical scenario has been reported among Asiatic children in both carriage and disease isolates, and frequently associated to PVL. Of major concern was the local emergence of methicillin resistance in ST121 isolates in these pediatric communities: one MRSA isolate, PVL-negative responsible for staphylococcal scalded skin syndrome in China and two MRSA, PVL-positive, SCC*mec* type V isolates responsible for osteomyelitis and soft tissue abscess, in Cambodia (7, 15). Moreover, single ST121 MRSA isolates were reported in China (http://saureus.mlst.net/) and the United States (31).

Table 1. Molecular characterization and clinical presentation of the clonal lineages found among the 38 *Staphylococcus aureus* isolates recovered during the 15-month study period.

No. of isolates	PFGE types (no. of subtypes)	*spa* type[a]	MLST[b]		SCC*mec* type	PVL	*agr* type	Related clone[c]	Clinical presentation (no. of isolates)
			ST	CC					
19	A (8)	t159	121	121	MSSA	-	IV		Cellulitis (9), cutaneous abscess (3), impetigo (1), wound (1), nasal swab (5)
6	B (4)	t318	30	30	MSSA	+	III	Southwest Pacific	Cutaneous abscess (6)
3	C (3)	t576	45	45	MSSA	-	I	Berlin	Cutaneous abscess (2), cellulitis (1)
2	D (1)	t535	5	5	IVc	-	II	Pediatric	Impetigo (1), bacteremia (1)
1	D (1)	t311	5	5	MSSA	+	II	Pediatric	Cutaneous abscess (1)
3	E (2)	t355	152	152	MSSA	+	I		Cutaneous abscess (1), cellulitis (1), adenophlegmon (1)
2	F (2)	t127	1	1	MSSA	+	III		Cutaneous abscess (1), pustule (1)
1	G	t044	80	80	IVc	+	III	European	Pustule (1)
1	H	t084	582	15	MSSA	-	IV		Impetigo (1)

[a] Ridom nomenclature (http://spaserver.ridom.de/).
[b] MLST – multilocus sequence typing, ST – sequence type; CC – clonal complex, defined by eBURST v3 assessed on 12 April 2010.
[d] Common designation of community - or hospital-associated MRSA or MSSA clones.

Retrospectively, all STs found in the present study (with the exception of ST152) have already been described in carriage among the Portuguese community in 1996, although with a different prevalence (4). Despite the small size of the collection, the major CA-MSSA clones described in Europe, ST1, ST5, ST30, and ST45 (19) were found in the pediatric Portuguese community (Table 1). Moreover, all clonal lineages, with the exception of PFGE H/t084/ST582/*agr*IV, were recently reported among a geographical and temporal diverse collection of 211 PVL-positive CA-MSSA (33). ST1/t127, the sixth most frequent type among MSSA isolates recovered in 26 European countries (19), was also found in the present collection. In the same work, Grundmann et al. showed that European MSSA isolates belonged to more diverse genetic backgrounds and have a wide geographical distribution compared to MRSA, which have a predominantly regional spread of a few pandemic clones (19).

A non-multiresistant antibiotic pattern is common in the present collection (Figure 1). Clindamycin and trimethoprim-sulfamethoxazole (SXT) are rational empiric choices for mild-to-moderate CA-MRSA infections (36). However, a positive D-test result in erythromycin-resistant isolates indicates that clindamycin resistance may emerge during therapy and, therefore, should not be prescribed (21, 22). The detection of clindamycin inducible resistance in all erythromycin-resistant isolates (n = 10) raises some concerns about the antibiotherapy available for the treatment of children, namely, since resistance to STX was found in three isolates from children with no previous hospitalization.

As far as we know, the single tetracycline-resistant strain, HFF189, was the first MRSA ST80 (known as the European CA-MRSA clone), PVL-positive described in Portugal. Nevertheless, since it was recovered from a periumbilical exudate of a neonate, the connection to the nosocomial setting could not be discarded, as ST80 PVL-positive isolates were already described as nosocomial isolates in the late 1990s (3). Regular surveillance studies in Portuguese hospitals (2), together with the present study seem to indicate that CA-MRSA-ST80 is not widely spread in Portugal in contrast to what was described in several other European countries (17, 38).

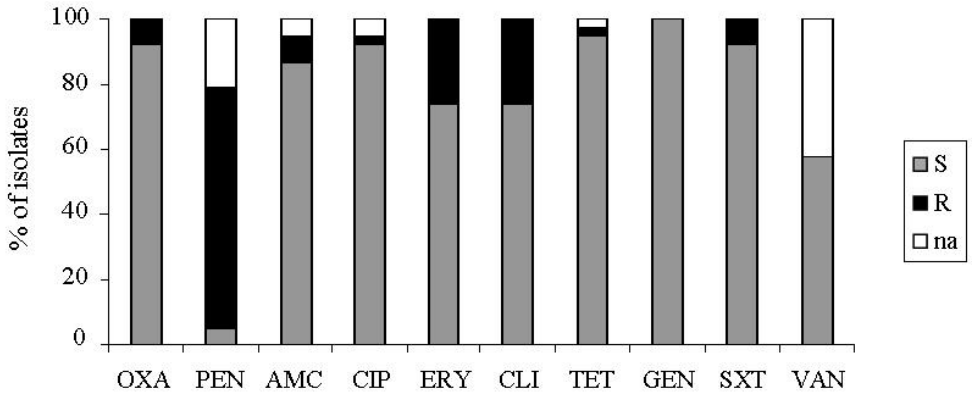

Figure 1. Antimicrobial susceptibility of the 38 *S. aureus* isolates to a panel of 10 antibiotics. Abbreviations: S, susceptible; R, resistant; na, not available; OXA, oxacillin; PEN, penicillin; AMC, amoxicillin-clavulanic acid, CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin. All the resistant phenotypes observed for clindamycin mean inducible resistance, determined by D-test (9).

Interestingly, the remaining two MRSA isolates, PVL-negative, showed ST5-SCC*mec* type IV typical of the Pediatric clone, described for the first time in a Portuguese pediatric hospital (35). The spread in the community of a typical HA-MRSA lineage raises some concern about the changing epidemiology of MRSA, and the blurring of the boundaries between the hospital and the community.

All of the isolates presented virulence determinants, namely, leukocidins (Figure 2). Recently, a mechanism of PVL-induced acute lung injury and lung inflammation in rabbit models resolved the controversy about the role of PVL as a key factor in *S. aureus* infection (12). In our study, isolates producing PVL or γ-hemolysin were mainly associated to cutaneous abscesses (p = 0.003 and p = 0.000, respectively), as has already been reported in recent studies in children infections (11, 40). Conversely, the production of β-hemolysin, ETA, or ETB seemed to be associated with cellulitis (p = 0.010, p = 0.003 and p = 0.038, respectively) (data not shown).

No significant positive association was found between socio-demographic data or possible risk factors for *S. aureus* infection, possibly due to the small dimension of the collection (data not shown).

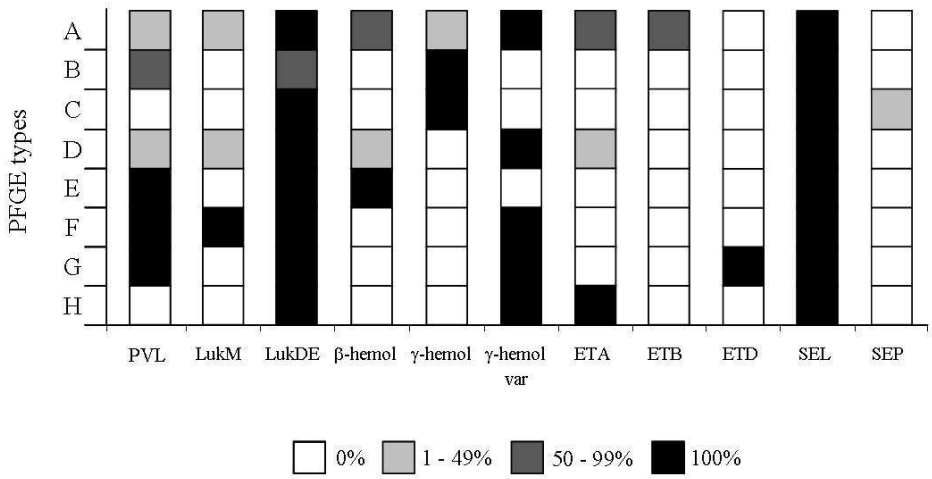

Figure 2. Virulence gene determinants of the eight clonal lineages. The totality of the isolates was tested for 11 virulence genes. Abbreviations: PVL, Panton Valentine leukocidin; LukM, leukocidin M; LukDE, leukocidins D and E; β-hemol, β-hemolysin; γ-hemol, γ-hemolysin; γ-hemol var, γ-hemolysin variant; ETA, ETB, ETD, exfoliative toxins A, B and D; SEL, SEP, staphylococcal enterotoxins L and P.

MSSA infections, independently of the PVL content, frequently show similar epidemiological and clinical characteristics to MRSA, but specific PVL-positive MSSA lineages are dynamically interrelated and recently reported as reservoirs of CA-MRSA (26, 33). Therefore, a regular surveillance of SSTI, namely, in children, is critical in order to predict and control the emergence of methicillin resistance and spread of staphylococcal infections in the community.

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T. Conceição performed the experimental work, analysis of the results and wrote the manuscript.

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Chapter IV

Unusually high prevalence of nosocomial Panton-Valentine leukocidin-positive *Staphylococcus aureus* isolates in Cape Verde islands

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ABSTRACT

Characterization of nosocomial methicillin-susceptible *Staphylococcus aureus* isolates from Cape Verde showed that (i) Panton-Valentine leukocidin genes were present in 35% of the isolates and (ii) half of the collection had the same genetic background as methicillin-resistant pandemic clones. Introduction of the staphylococcal chromosome cassette *mec* (SCC*mec*) into virulent and epidemic isolates could pose serious threats to public health.

RESULTS and DISCUSSION

Staphylococcus aureus constitutes a major public health threat, and methicillin-resistant *S. aureus* (MRSA) is currently the most commonly identified antibiotic-resistant pathogen in many parts of the world. Concerning the African continent, although there is still a lack of epidemiologic data, MRSA was reported as a problem in different countries, including South Africa (6), Zimbabwe (16), Kenya (13), Ethiopia (29), Egypt (9), Senegal (27), and Ivory Coast (4). In contrast, the prevalence of MRSA was very low or nonexistent in Somalia (23), Nigeria (1), and Tanzania (30).

In a previous study we determined the rates of nosocomial colonization by staphylococci in Cape Verde islands and the antimicrobial susceptibility profiles (3). The *S. aureus* carriage rates were around 41%, and all isolates were susceptible to methicillin (3). The aim of the present study was to obtain insights into the methicillin-susceptible *S. aureus* (MSSA) genetic population, including the detection of virulence determinants, in a country where at least in a particular period the prevalence of MRSA in colonization samples seemed to be null.

The MSSA isolates included in this work were collected during a surveillance study in two hospitals on the two most populated islands of the Cape Verde archipelago (3). Hospital Dr. Agostinho Neto is located in Praia, on São Tiago, and Hospital Baptista de Sousa is in Mindelo, on São Vicente. The isolates represented colonization samples from patients or health care workers and were recovered from nasal or wound swabs. Five isolates from the previous study were not viable when cultured from the frozen stocks and could not be included in the present work.

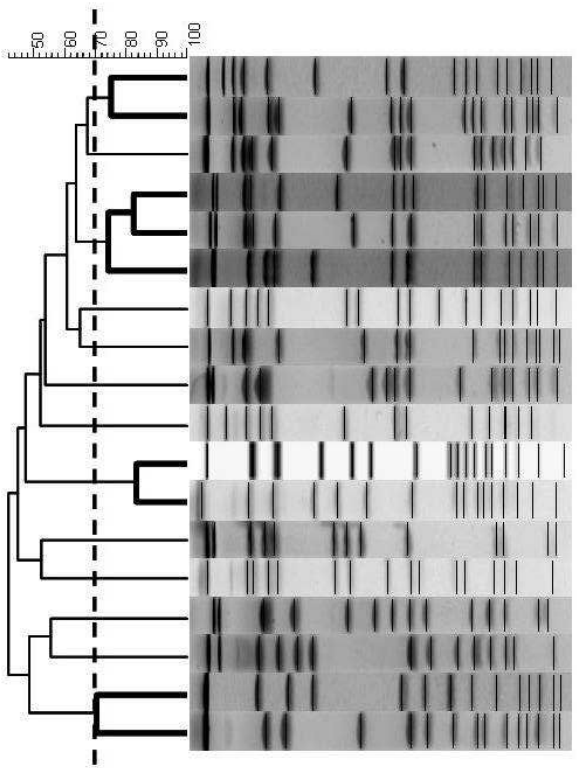
Pulsed-field gel electrophoresis (PFGE) was performed after SmaI digestion as described by Chung et al. (7). The resulting band patterns were analyzed by visual inspection followed by analysis with BioNumerics software (version 4.0; Applied Maths, Gent, Belgium) for relatedness evaluation. Dendrograms were generated from similarity matrixes calculated with the Jaccard coefficient, and patterns were clustered by the unweighted pair group method with arithmetic mean using an optimization of 1.5% and a tolerance of 1.3%. Profiles

with more than 70% similarity were considered closely related. *spa* typing was performed as previously described (17, 26). The *spa* types were assigned through the eGenomics and Ridom (http://www.ridom.de/spaserver/) (12) web servers. *spa* types with similar repeat profiles were grouped together as part of the same lineage (*spa* lineage); lineages are identified by the same capital letter.

Multilocus sequence typing (MLST) was carried out as described by Enright et al. (10), with the exception that primer *arcC*F2 (5'-CCT TTA TTT GAT TCA CCA GCG-3') was used (8). *spa* typing and MLST PCR products were purified with a Wizard PCR Preps purification system (Promega, Madison, WI) and used as templates for sequencing of both strands at Macrogen (Seoul, South Korea). MLST alleles and sequence types (STs) were identified using the MLST database (http://www.mlst.net), which was also used to search for earlier reports on the STs identified during the present study.

The presence of the Panton-Valentine leukocidin (PVL) and the exfoliative toxin D (ETD) genes, i.e., *lukS-PV* and *lukF-PV* and *etd*, respectively, was determined by PCR as described previously (15, 19).

The characterization of 63 MSSA strains by PFGE resulted in the definition of 13 different genotypes (Figure 1). Representatives of these 13 PFGE patterns (17 isolates) were characterized by *spa* typing and classified into 17 *spa* types and 12 *spa* lineages. MLST was performed on single isolates of each PFGE pattern and/or *spa* lineage identified (13 isolates). All PFGE types belonged to a different ST, confirming the existence of 13 distinct clones among the 63 MSSA isolates, whose main characteristics are shown in Figure 1.



Isolate	PFGE type	spa lineage	*spa* profile	*spa* type	ST	PVL / *etd*
HPV107	**Iberian**	**J**	YHFGFMBQBLO	4 /t051	247	- / -
CV58	J13	J	WMBQBLO	651 /t301	683	- / -
CV41	E2	E	UJGFGMDMGGM	193 /t148	72	- / -
HDE288	**Pediatric**	**C**	TJMBDMGMK	45 /t311	5	- / -
CV66	C1	C	TJMBMDMGMK	2 /t002	5	- / -
BK2464	**NY/Japan**	**C**	TJMBMDMGMK	2 /t002	5	- / -
CV73	G7	G	UJFKBPE	35 /t127	1	+ / -
CV87	O	O	ZFGMDMGGMK	617 /t941	668	- / -
CV17	K1	K	UJGBBGGJAGJ	21 /t084	15	- / -
CV161	U1	U	UJGFMBBPB	92 /t359	669	- / -
Berlin IV	**Berlin**	**H**	A2AKEEMBKB	15 /t004	45	- / -
CV81	H3	H	XKAKBBEMBKE	472 /t861	508	- / -
CV15	F8	F	I2Z2GMJH2T2M	615 /t940	121	+ / -
CV168	S7	O	ZFGMDMGGM	618 /t349	25	+ / +
CV55	V	V	XKBQBBMM	208 /t937	398	- / +
CV18	Z1	Z	ZKBGBG	616 /t939	45	- / -
96/32010	**EMRSA-16**	**A**	WGKAKAOMQQQ	16 /t018	36	- / -
CV11	A17	A	WAKAOMQ	650 /t942	30	+ / -

Figure 1. Molecular characterization of MSSA strains and comparison with MRSA pandemic clones. On the left is a dendrogram showing the estimated relationships of PFGE types based on Bionumerics analysis, including representatives of five international pandemic MRSA clones (in bold). (a) The *spa* type was assigned through both the Egenomics and the Ridom web servers.

The genetic studies on the clonal population of the MSSA isolates in Cape Verde demonstrated that some clones were very successful. Clone U (*spa* type 92/ t359 or related, ST669) was represented by 19% of the isolates (*n* = 12), whereas the second and third most prevalent clones, clone J (*spa* type 651/ t301 or related, ST683) and clone A (*spa* type 650/ t942 or related, ST30), had incidences of 16% (*n* = 10) and 14% (*n* = 9), respectively. In addition, some of these clones have the capacity to disseminate between islands: 8 of the 13 clones occurred in both hospitals (Figure 2). However, the most successful clone, clone U, was identified in HBS only.

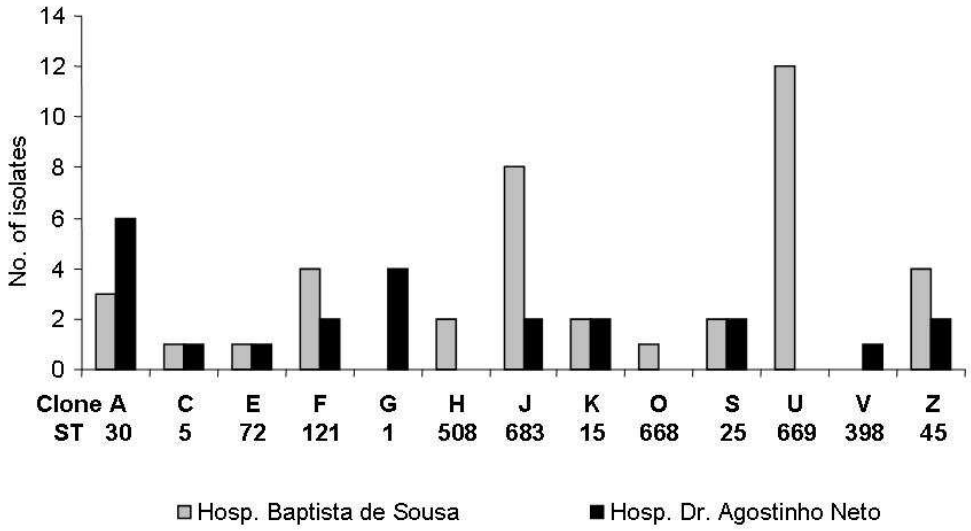


Figure 2. Clonal distribution between Hospital Baptista de Sousa (Praia, São Tiago) and Hospital Dr. Agostinho Neto (Mindelo, São Vicente).

Although no MRSA strains have been found in Cape Verde, about half of the isolates (46%, *n* = 29) showed a genetic background identical (same ST) or very similar (single-locus variant [SLV] or double-locus variant [DLV]) to the genotypes of pandemic MRSA clones. For instance, clone J (ST683) is a DLV of the Iberian MRSA (ST247), clone A (ST 30) is an SLV of EMRSA-16 (ST36), and clone C (ST5) has a background identical to the New York/ Japan and Pediatric (ST5) clones. Moreover, clone H (ST508) is an SLV of and clone Z (ST45) shares

the same ST as the Berlin clone (ST45) (Figure 1). The fact that some of the major MSSA clones established in Cape Verde correspond to internationally successful MRSA clones provides evidence that the emergence of MRSA isolates in an environment is not exclusively dependent on the presence of successful MSSA lineages. A similar observation was recently published from Portugal (2).

Although most MSSA clones in Cape Verde are recognized as international clonal types, three STs found in the present study have not been described before (STs 668, 669, and 683). Interestingly, ST398, which was detected in a single isolate in the present study, was recently reported to be resistant to digestion with SmaI restriction endonuclease (33) and seems to be associated with pigs and pig farmers (5, 33). Whether the isolate from Cape Verde was pig related is unknown. However, there were no signs of resistance to SmaI digestion, and the isolate was classified as PFGE type V.

A total of 22 (35%) and 5 (8%) isolates tested positive by PCR amplification for the PVL and ETD genes, respectively. All but one isolate belonging to STs 30 (*n* = 8), 121 (*n* = 6), 1 (*n* = 4), and 25 (*n* = 4) were PVL positive, whereas the five ETD-positive isolates belonged to ST25 (*n* = 4) and ST398 (*n* = 1). Out of the 22 PVL-positive isolates that were almost equally distributed among patients and health care workers, four were recovered from wound infections, whereas 18 were obtained from nasal samples. We found a proportion of PVL-positive MSSA isolates that was fairly high (35%) compared with data in studies from other countries. Melles et al. (21) found a PVL prevalence of only 0.6% in a large (*n* = 829) Dutch collection of carriage isolates, whereas Kuehnert et al. (18) reported the presence of PVL in 1% of 297 American MSSA nasal isolates, and Holmes et al. (14) found only 8 PVL-positive isolates (1.6%) among 515 isolates of *S. aureus* from different sites of infection. An additional study demonstrated that PVL genes were very rare in Germany in both nasal and blood isolates (32). Moreover, the PVL gene has been reported to be more common in MRSA isolates than in MSSA isolates (18, 20), particularly among community-acquired MRSA strains of STs 1, 8, 30, 59, 80, and 93 (31).

In the present study, the PVL genes were detected in all isolates belonging to clones A-ST30 (with the exception of one isolate), F-ST121, G-ST1, and S-ST25, whereas *etd* was present in all isolates of clones S and V-ST398. A previous study comparing the genetic backgrounds of MSSA and MRSA in Portugal showed some MSSA clonal overlapping between Portugal and Cape Verde. Although the four PVL-positive clonal types detected in Cape Verde had been identified among the Portuguese MSSA nosocomial and/or community population, the cytotoxin was found exclusively in clone F-ST121 and in a very restricted number of Portuguese isolates (2 out of 19 isolates) (2). Clones ST1 and ST30 PVL-positive have been described as the most frequent community-acquired MRSA clones in the United States and Oceania, respectively (31). ST121 has been detected among isolates carrying PVL genes in England and Wales (14), and ST25 was recently reported as a highly transmissible clone associated with colonization and infection in the niche of AIDS-infected patients with a history of drug use (11). It is known that the genes that encode PVL are carried by different temperate phages and that *S. aureus* strains are usually lysogenized by a phage(s) (22). Our results suggest that hospitals in Cape Verde may represent a reservoir of PVL-positive clones. However, the reason why MSSA strains from Cape Verde seem to be more prone to PVL-converting phage infection is currently unclear. This study indicates that the acquisition of virulence might confer a greater advantage to *S. aureus* than antibiotic resistance genes in a country and period in which the use of antibiotics is very limited.

Interestingly, clone A is closely related to phage type 80/81 strains, which became pandemic during the 1950s, causing a high frequency of skin lesions, sepsis, and pneumonia in children and young adults in hospitals and the community (25). The phage type 80/81 clone is ST30, carries the PVL genes, and has been suggested to be the ancestor of the community-acquired PVL-positive southwest Pacific MRSA clone (ST30, SCC*mec* IV) (24). On the other hand, Taneike et al. recently reported that the MRSA isolates that caused nosocomial outbreaks in Japan in the 1980s were ST30 and PVL positive (28). This clone was replaced by the PVL-negative New York/ Japan MRSA ST5 clone in the 1990s

and now seems to be re-emerging in the community after having lost some virulence genes (28).

In summary, the genetic backgrounds corresponding to five pandemic MRSA clones were represented among the MSSA isolates from Cape Verde, and the genes that code for PVL were detected in a high proportion of strains with different genetic backgrounds. Although a fitness cost associated with the maintenance of both SCC*mec* and the PVL genes may exist, we should be aware of the eventual emergence of MRSA strains by the introduction of SCC*mec* into highly virulent and epidemic MSSA backgrounds. This could pose an additional threat to public health, warranting continuing surveillance studies, namely in African countries.

Post publication note of the authors:

Soon after the publication of the present data, an occasional review of the MLST results showed that the single PFGE type V, *spa* type 208/ t937, ETD positive isolate (CV55) was incorrectly assigned to ST398. In fact, the allelic sequence of this isolate (3-37-19-2-20-26-32) corresponds to the ST291, a single locus variant of ST398 (3-35-19-2-20-26-32). Therefore, in the previous text it should be read ST291 instead of ST398, and the clone considered as a singleton in the collection.

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T. Conceição performed the experimental work and the analysis of results.

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Chapter V

Prevalence and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Atlantic Azores islands: predominance of SCC*mec* types IV, V and VI

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ABSTRACT

In order to obtain insights into the methicillin-resistant *Staphylococcus aureus* (MRSA) population structure in the Azores archipelago, 106 MRSA isolates were collected from patients attending an Azorean central hospital between January 2007 and February 2008. Antimicrobial resistance was determined for all isolates. Molecular typing was performed by pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST), staphylococcal chromosome cassette *mec* (SCC*mec*) typing and the presence of Panton–Valentine leukocidin (PVL). The majority of the isolates (87%, *n* = 92) belonged to the EMRSA-15 clone (ST22, SCC*mec*-IVh), followed by the Pediatric clone (ST5-VI/IVc) (11%, *n* = 12). The Berlin clone (ST45-IVa) and a new clone (*spa* type t1839, ST1339 and SCC*mec* V variant) were represented by single isolates. All of the isolates carried SCC*mec* types IV, V or VI and a non-multiresistant antibiotic profile, resembling the currently emerging community MRSA. Moreover, PVL was described for the first time to be associated with the Pediatric clone carrying SCC*mec* type VI. We provided the first description of the population structure of MRSA in the Azores islands, which seems to be shaped by genetic events occurring locally, as well as by the regular population exchange between the islands, continental Portugal, the United Kingdom and the United States.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen associated with both nosocomial- and community-acquired infections (hospital-acquired [HA] MRSA and CA-MRSA, respectively) (14). MRSA are believed to have emerged by the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*), the mobile genetic element carrying the determinant of methicillin resistance (*mecA*) into methicillin-susceptible *S. aureus* (MSSA). Eight different SCC*mec* types (I to VIII) have been described so far in *S. aureus*, differing in their structure and size. Epidemiological studies using molecular typing methods showed that the massive geographical spread of MRSA in hospitals results from the dissemination of a few highly epidemic clones. These major epidemic clones resulted from a limited number of independent acquisitions of SCC*mec* into well-defined genetic backgrounds: (i) New York/Japan clone, sequence type (ST) 5, SCC*mec* II; (ii) Pediatric clone, ST5-IV/VI; (iii) Brazilian clone, ST239-III/IIIA; (iv) Iberian clone, ST247-IA; (v) EMRSA-15, ST22-IV; (vi) EMRSA-16, ST36-II; and (vii) Berlin clone, ST45-IV (12, 29, 34).

The MRSA prevalence in European hospitals varies from less than 3.0% in the Nordic Countries and The Netherlands to over 50% in southern European countries and the United Kingdom. Despite the significant decrease in MRSA incidence in a few countries (France, Slovenia, Belgium, Cyprus and Turkey) due to improved infection control, the incidence has increased in other countries, including Portugal, where the MRSA proportion is, nowadays, over 50%, being one of the highest in Europe (2, 14).

Surveillance studies have been conducted in Portugal since the early 1990s, showing temporal waves of MRSA clonal prevalence. In the most recent survey conducted in 2006, EMRSA-15 (ST22-IVh) was found to be the most prevalent clone in Portuguese hospitals, followed by the New York/Japan clone (ST5-II) (2, 5).

Information about the epidemiology of CA-MRSA is still scarce and non-existent in Portugal. Unlike HA-MRSA, CA-MRSA are usually only resistant to beta-lactams and one or two additional antimicrobial classes, and carry mainly

SCC*mec* types IV and V, the smallest in size, which are believed to be more mobile. CA-MRSA frequently harbour Panton-Valentine leukocidin (PVL), but the toxin has also been detected in the hospital environment (16, 42). Similarly to HA-MRSA, CA-MRSA epidemic clones have been described (7). However, a much larger genetic diversity in the genetic backgrounds of CA-MRSA has been observed, suggesting a high number of SCC*mec* acquisitions in the community. Recent data indicates that MRSA epidemiology is suffering a new change and that the boundaries between the hospital and the community are blurring. As a result, clones traditionally linked to the hospital, like the EMRSA-15 clone, were found to be aetiologic agents of infection in the community (30) and clones specific to the community were observed as causing infections in hospitals, as is the case of the USA300 strain (38, 40). A common trait to these clones that can survive in both environments appears to be the presence of SCC*mec* type IV that is, nowadays, prevalent among the current overall MRSA population (2, 4, 5, 20, 43).

Whereas the epidemiology of nosocomial MRSA has been very well characterised in the Portuguese continental territory, there is no information concerning the prevalence and clonality of MRSA in the Portuguese islands, namely in the Azores archipelago.

The aim of the present study was to identify the MRSA clonal types currently circulating in the Azores archipelago and compare them with the major MRSA clones described in continental Portugal, Europe and the United States.

MATERIAL and METHODS

Hospital setting

Hospital do Divino Espírito Santo (HDES) is a 390-bed central hospital located in Ponta Delgada, São Miguel island, that services an outpatient population of 138,000 inhabitants, from the two most populous Azorean islands, S. Miguel and Santa Maria. The hospital, the largest in the archipelago, includes medicine and surgery as principal wards, and a large outpatient ambulatory unit.

Bacterial isolates

Between January 2007 and February 2008, a total of 106 MRSA isolates were collected from both inpatients (*n* = 93, 87.7% of the isolates) and outpatients (*n* = 13, 12.3% of the isolates) attending HDES. The collection included single-patient isolates recovered from several sources, including sputum (*n* = 35, 33%), urine (*n* = 22, 21%), swabs from various origins (*n* = 19, 18%), exudates from wounds, one abscess and one ear infection (*n* = 16, 15%), blood (*n* = 10, 9.5%), catheters (*n* = 2, 1.9%) and auricular and peritoneal fluids (*n* = 1, 0.9% each). Most of the isolates (95%, *n* = 101) were from infection. The majority of the patients (73.5%) were inpatients hospitalised in three wards: medicine (*n* = 42, 39.6%), surgery (*n* = 24, 22.6%) and pneumology (*n* = 12, 11.3%). Among the 13 outpatients, three attended the haemodialysis unit.

Antimicrobial susceptibility testing and *mecA* detection

Susceptibility testing was performed with the semi-automatic Vitek system (bioMérieux, SA, France) according to the manufacturer's instructions for a panel of 12 antibiotics: ciprofloxacin, clindamycin, erythromycin, gentamicin, linezolid, oxacillin, penicillin, rifampicin, trimethoprim-sulfamethoxazole, teicoplanin, tetracycline and vancomycin. Bacterial isolates were considered to belong to different antibiotypes if at least one difference was observed in the antibiotic resistance profile.

The presence of the *mecA* gene was confirmed in all oxacillin-resistant isolates by polymerase chain reaction (PCR) (31).

PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as described by Chung et al. (8) on all 106 isolates. The resulting SmaI restriction patterns were analysed by both visual inspection using the criteria of McDougal et al. (23) and automatically with the BioNumerics software version 4.61 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated as previously described (15) using an optimisation of 0.50% and a tolerance of 1.25%. A similarity coefficient of 80% was used to define the PFGE type clusters (15).

***spa* typing and MLST**

spa typing was carried out on at least one representative of each PFGE subtype (*n* = 26) and *spa* types were assigned through the Ridom web server (http://spaserver.ridom.de) (1). Multilocus sequence typing (MLST) was conducted as described (10) for selected strains previously typed by PFGE and *spa* typing. The allelic profiles and sequence types (ST) were defined using the MLST database (http://www.mlst.net).

SCC*mec* and *ccr* typing

SCC*mec* was typed by the multiplex PCR strategy described by Milheiriço et al. (25). Additionally, all type IV isolates were further subtyped as previously described (24). Type VI isolates were confirmed by amplification of the cassette chromosome recombinase genes *ccrAB4* (32). Type V was confirmed by PCR amplification of *ccrC* and *mec* complex C, as previously described (19, 31). Non-typeable isolates by the methods mentioned above were characterised by *ccrB* sequencing (33).

PVL detection

All 106 MRSA isolates were screened for the presence of PVL *lukS*/*lukF* genes by PCR, as described previously (22). Positive results were confirmed by sequencing of the PCR amplicon.

RESULTS

Antimicrobial susceptibility

The 106 isolates were assigned to eight different antibiotypes (Table 1) with a prevalent non-multiresistant pattern.

The majority of the isolates (*n* = 91, 85.9%) showed antibiotype 1 (resistance to oxacillin, penicillin, ciprofloxacin and erythromycin) or 2 (resistance to oxacillin, penicillin and ciprofloxacin). The isolate with antibiotype 7 was unique in showing resistance to gentamicin. Antibiotype 8, which showed resistance to six different antimicrobials, included a single isolate.

All 106 isolates harboured the *mecA* gene and, therefore, were classified as MRSA.

Table 1. Resistance profile of the 108 MRSA isolates and associated PFGE types

Antibio type	Resistance profile[a]												Total nº of isolates (%)	PFGE types (subtypes)
	OXA	PEN	CIP	ERY	CLI	RIF	GEN	LZD	SXT	TEC	TET	VAN		
1	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	S	67 (63.2)	A (1-9); B (1, 2); D (1)
2	**R**	**R**	**R**	S	S	S	S	S	S	S	S	S	24 (22.7)	A (1-6); B (1, 4); C (1)
3	**R**	**R**	S	**R**	S	S	S	S	S	S	S	S	7 (6.6)	A (1, 2, 5); B (1)
4	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	3 (2.8)	A (1)
5	**R**	**R**	S	S	S	S	S	S	S	S	S	S	2 (1.9)	A (4); B (3)
6	**R**	**R**	**R**	S	S	**R**	S	S	S	S	S	S	1 (0.9)	A (1)
7	**R**	**R**	S	S	**I**	**R**	**R**	S	S	S	S	S	1 (0.9)	B (1)
8	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	1 (0.9)	B (1)

[a] S, Susceptible; I, intermediate; R, resistant. Antibiotic abbreviations: OXA, oxacillin; PEN, penicillin; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; RIF, rifampicin; GEN, gentamicin; LZD, linezolid; SXT, trimethoprim-sulfamethoxazole; TEC, teicoplanin; TET, tetracycline; VAN, vancomycin.

Predominance of EMRSA-15 clone

The characterisation of the 106 MRSA strains by PFGE, *spa* typing, MLST and SCC*mec* typing clustered the isolates into four clonal types (Figure 1). The majority of the isolates (*n* = 92, 87%) belonged to PFGE type A. PFGE type B included 11% (*n* = 12) of the isolates and types C and D were represented by single isolates only (1% each).

PFGE type A was subdivided into nine subtypes; subtypes A1 (*n* = 40) and A2 (*n* = 22) represented 67% of all type A isolates. The representative isolate of

PFGE type A (subtype A1) showed *spa* type t032, ST22 and SCC*mec* type IVh, which are characteristics of the internationally disseminated EMRSA-15 clone (27). Interestingly, subtype A4 showed the same restriction profile as the EMRSA-15 clone reference strain, HAR22, but a different *spa* type (t032), including one additional repeat sequence (Figure 1). All representative isolates of type A shared the same *spa* type t032, except one isolate (t910), which differs from t032 by the deletion of five repeats. HDES85 was the unique isolate of PFGE type A1 defined as non-typeable by *spa* typing, since no PCR product was obtained. Another single type A1 isolate, HDES73, showed a SCC*mec* cassette type IV non-subtypeable. PFGE type A included isolates from six out of the eight antibiotypes described, but 95% (n = 53) of the isolates belonged to antibiotypes 1 and 2 (Table 1).

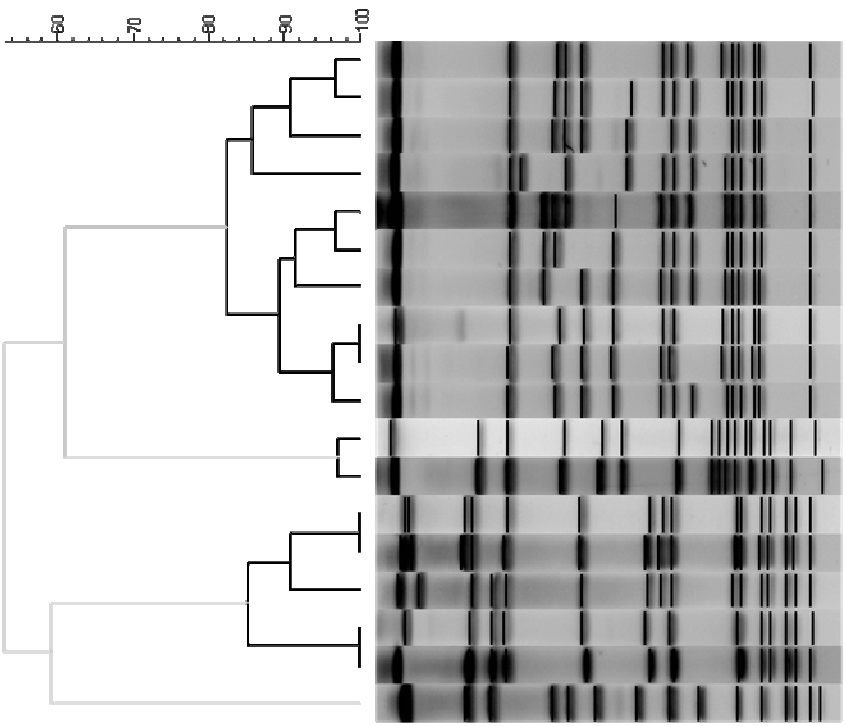
Evidence of horizontal gene transfer among strains of the Pediatric clone PFGE type B was the second most common PFGE type and included three subtypes, of which B1 represented 75% (n = 9) of type B isolates (Figure 1). Subtypes B1 and B2 showed ST5, SCC*mec* VI and *spa* types t062, t2049, t2724 (B1)/t311 (B2), which are typical of the Pediatric clone. SCC*mec* type VI was confirmed in five isolates by amplification of the characteristic *ccrAB4* genes. The remaining type VI isolates did not amplify the *ccrAB4* genes by uniplex PCR but showed a *ccrB* sequence with 100% homology with the Pediatric clone reference strain HDE288 (37). Interestingly, subtype B3 carried SCC*mec* type IVc instead of the SCC*mec* type VI, which may indicate the occurrence of independent acquisition of SCC*mec* in the Pediatric clone background.

Moreover, we found that three isolates belonging to PFGE subtype B1 (ST5-VI) carried the PVL genes.

The remaining two isolates belonged to PFGE types C and D and were, respectively, recovered from an outpatient and an inpatient. PFGE C isolate, *spa* type t004 and ST45-IVa showed a single band difference relative to HAR38, the prototype isolate of the Berlin clone (*spa* type t004, ST45-IVa) (9, 28). PFGE type D isolate showed *spa* type t1839, a novel sequence type (ST1339) not related to any major clonal complex (CC) and a variant of SCC*mec* type V.

High genetic diversity and high frequency of SCC*mec* types IV, V and VI

SCC*mec* type IV was found to be the prevalent type (*n* = 13) among the 24 isolates tested. Three different SCC*mec* IV subtypes were found in this collection, associated to three unrelated genetic backgrounds (ST5: SCC*mec* IVc, ST22: SCC*mec* IVh/IVnon-subtypeable, and ST45: SCC*mec* IVa). SCC*mec* type VI was the second most prevalent cassette type, which appeared to be associated to isolates with PFGE types B1 and B2. A variant of SCC*mec* type V was shown on a single isolate that harboured the *mec* complex C and *ccrC* genes, but missed the specific type V J1 region. No isolates carrying SCC*mec* types I, II or III were identified in this collection.



Strain	PFGE type	*spa* type	ST	SCC*mec* type	PVL	Antibiotype
HDES57	A1	t032	22	IVh	-	1
HDES96	A9	t032		IVh	-	1
HDES54	A3	t032		IVh	-	1
HDES102	A5	t032		IVh	-	1
HDES13	A7	t032		IVh	-	1
HDES51	A6	t032		IVh	-	1
HDES65	A8	t032		IVh	-	1
HDES47	A4	t032		IVh	-	1
HAR22	EMRSA-15	t022	22	IVh	-	
HDES52	A2	t910		IVh	-	1
HAR38	Berlin clone	t004	45	IVa	-	
HDES79	C1	t004	45	IVa	-	2
HDE288	Pediatric clone	t311	5	VI	-	
HDES1	B2	t311	5	VI	-	1
HDES26	B1	t062	5	VI	+	2
HDES93	B3	t002		IVc	-	2
USA800	Pediatric clone	t002	5	IV	-	
HDES41	D1	t1839	1339	Vvar	-	1

Figure 1. Molecular characterisation of representative methicillin-resistant Staphylococcus aureus (MRSA) isolates of each pulsed-field gel electrophoresis (PFGE) subtype and comparison with MRSA pandemic clones

DISCUSSION

In order to describe the MRSA population structure in the Atlantic Azores islands, a collection of 106 isolates recovered during a 13-month period was characterised by a combination of up-to-date molecular typing methods. The analysis showed the predominance of two major international MRSA clones: the EMRSA-15 clone and the Pediatric clone.

Massive dominance of the EMRSA-15 clone

In the present study, 87% of the isolates showed molecular characteristics of the highly internationally disseminated EMRSA-15 clone (ST22-IVh) (27), including a common *spa* type t032. In addition, a single PFGE type A2 isolate showed t910, a *spa* type already reported in a CC22 isolate from Germany (41). This fact is not surprising, since the *spa* gene is a variable repeat region prone to the introduction or deletion of repeats. Regional *spa* clusters frequently include highly related *spa* types characterising clonal related isolates, which translates a local evolution of the *spa* locus (16, 17). Unusually, a non-typeable isolate was found in the collection, which should be due to a mutation or insertion/deletion in *spa* rather than non-existence of the gene (6).

Interestingly, the majority of the EMRSA-15 isolates described in this study were recovered from sources other than blood (sputum, swabs, exudates and urine), corresponding to the major pathologies presented by the patients attending HDES (respiratory tract and skin related). The non-multiresistant EMRSA-15 clone emerged in the United Kingdom in 1991 and rapidly became a dominant clone worldwide, particularly in Europe (16, 21). Recently, an EMRSA-15 variant was reported in central United States (48). In the Azores islands, the clear dominance of the EMRSA-15 clone is, in part, in agreement with the situation in continental Portugal (2, 5). In fact, in a study involving isolates recovered in the same period from 11 hospitals scattered over continental Portugal, EMRSA-15 was found to be the most prevalent clone. However, and in contrast with the present study, the New York/ Japan clone (ST5-II) was shown to represent a new wave of MRSA in the country, whereas the Pediatric clone (ST5-VI/IV) was exceedingly rare (2). In

the Portuguese neighbouring country Spain, EMRSA-15 was found in continental territory in co-existence with EMRSA-16 and two CC5 clones (ST125-IV and ST146-IV), but not as a major clone as in the Spanish islands (Majorca and Canarias) and as in our study (4, 35, 44). The frequent touristic flux from the United Kingdom, hypothesised as the cause of the importation of EMRSA-15 to the Mediterranean island of Malta (39), could also support, in part, the emergence of this clone in the most touristic Azores islands. In parallel, the regular exchange of inhabitants between the islands and the continent seem to be an important vehicle of clonal importation.

Emergence of PVL and SCC*mec* variability: evidence for local evolution

Recent studies revealed that the Pediatric MRSA clone, isolated for the first time in 1992 in a Portuguese hospital (37), was no longer present in that hospital in 2006 (2). However, it appeared in the present collection as the second major clone.

Interestingly, in this study, we observed that strains of the Pediatric clone differing in three to five bands in PFGE pattern, isolated in the same hospital, carried either the typical SCC*mec* types VI or IVc. ST5-VI MRSA isolates, which had been restricted to isolates from continental Portugal, were recently found in a collection of nosocomial isolates recovered from 23 hospitals located throughout France (11, 32). The hypothesis of a recent introduction of this clone from continental Portugal seems to be quite remote, since, in a recent national study, this clone was isolated from a single patient only in a hospital in the South of Portugal (2). On the other hand, the Azores islands have a high emigrant community in the United States, who frequently travel to the national territory, constituting also possible vehicles of dissemination of the Pediatric clone strains already reported in this country.

ST5 lineage is known to frequently acquire mobile genetic elements, such as different SCC*mec*, antibiotic resistance and toxin genes (13, 29). In a recent study, Nübel et al. showed that at least 23 SCC*mec* acquisitions events have occurred within ST5 lineage, on multiple and independent occasions, showing evidence that the MRSA emerged in numerous circumstances and in distinct

locations rather than by the geographic dispersal of isolates (29). Moreover, besides being originally identified in a community-associated USA400 strain, the first introduction of SCC*mec* IV in *S. aureus* might have occurred in the ST5 Pediatric clone background that was circulating in hospitals in the 1990s (7). Similarly, our results suggest that SCC*mec* cassettes VI and IVc might have been introduced *de novo* and recently in these ST5 strains and that the Pediatric clone may be evolving locally. Another observation that supports the hypothesis of recent local evolution of the Pediatric clone is the presence of PVL-positive and PVL-negative strains with closely related PFGE profiles. Noteworthy, this is the first report of PVL among isolates belonging to the SCC*mec* type VI Pediatric clone. The introduction of the PVL genes in a genetic background with such a high epidemic potential is of particular concern. However, we cannot exclude the reverse hypothesis of a local evolution through the acquisition of SCC*mec* type VI by a PVL-positive MSSA. Only the analysis of the genetic background of susceptible *S. aureus* in the Azores islands would clarify the chronological acquisition of PVL and SCC*mec* in these Pediatric clone strains.

The Berlin MRSA clone (ST45-IVa), first observed in Berlin hospitals in 1993, is currently spread in some European countries (16, 45), showing a high epidemic potential not only in the hospital environment but also in the community (36, 47). This clone was found in a single MRSA isolate in the Azores island collection. The same genetic background (ST45) had been previously described in Portuguese hospitals, but exclusively among MSSA isolates (3). The collection of a single MRSA isolate carrying SCC*mec* IVa suggests another *de novo* SCC*mec* acquisition, probably in an already established MSSA clone.

Likewise, the introduction of a variant of SCC*mec* type V seems to have occurred in an isolate with *spa* type t1839 and a novel sequence type (ST1339) that is a singleton. This strain is not related to the single SCC*mec* type V variant isolate (ST45) previously reported in Portugal (2). Interestingly, this isolate was recovered from a patient within the first 48 h of hospitalisation, from a skin and soft tissue infection (erysipelas), the main source of CA-MRSA isolates. However, the isolate could not be considered as having a community origin, since no additional information regarding patient risk factors was available.

Emergence of HA-MRSA isolates with community traits

SCC*mec* type IV is one of the most frequent SCC*mec* types found in the community and is, nowadays, becoming the predominant type also in the nosocomial setting (2, 4, 5, 7, 20, 43, 46). Moreover, this SCC*mec* type has been usually associated to a pattern of susceptibility to almost all antimicrobial classes. These same characteristics were observed in isolates from our study: SCC*mec* type IV was found to be the most frequent SCC*mec* type and all isolates were highly susceptible. At least three different SCC*mec* subtypes were identified (IVh: EMRSA-15, IVc: Pediatric clone, IVa: Berlin clone), showing evidence of a high genetic diversity among the pool of SCC*mec* type IV in the Azores islands. Interestingly, the detection of one isolate belonging to the EMRSA-15 clone, SCC*mec* type IV non-subtypeable (only positive for the *ccrB2* allele in the subtyping multiplex), suggests the presence of a new uncharacterised SCC*mec* type IV subtype in addition to the ones already described (24).

Besides SCC*mec* type IV, the only SCC*mec* types found among the whole collection were the small SCC*mec* V and VI, reflecting the actual tendency for the dominance of low resistant and fitness cost clones within the hospital setting, which is parallel with what is observed in the community environment (7). The introduction of well-established community isolates into the hospital setting is not new, as observed in Taiwan, where the ST59 clonal type, highly prevalent in the community, emerged in the hospital setting as the second most prevalent clone (18, 26, 35). Although ST22-IV EMRSA-15 related isolates as well as ST5-IV were already reported in the community (30, 46), no conclusions can be drawn for the flow direction in the present study. Even if a high percentage (30%) of the isolates studied was recovered within the first 48 h of admission or in an outpatient unit, no data of previous hospital contact was available for the patients.

In the present study, we provided the first description of the population structure of MRSA in the Azores islands and showed a prevalence of the epidemic EMRSA-15 clone in co-existence with the Pediatric clone. Our findings were in line with recent reports from continental Portuguese and European hospitals. The horizontal gene transfer and the *de novo* acquisition of mobile genetic elements seem to have contributed to the local genetic diversity and clonal evolution.

Moreover, the population exchange between the islands and continental Portugal or the United States, in addition to the high flux of tourists, particularly from the United Kingdom, may explain the dominance and spread of the EMRSA-15 and Pediatric clones. Further surveillance studies concerning both HA-MRSA and MSSA populations and CA-MRSA in the geographically restricted Azores islands will be fundamental to support the *S. aureus* localised evolution, as well as to understand the links between the hospital and the community.

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T.Conceição performed the experimental work (except the PFGE typing in half of the isolates, which was performed by A. Tavares), analysed the results and wrote the manuscript.

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Chapter VI

Replacement of methicillin-resistant *Staphylococcus aureus* clones in Hungary over time: a 10-year surveillance study

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ABSTRACT

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in Hungary has been increasing and is now close to 20% among invasive isolates of *S. aureus*. In order to understand the evolution of MRSA in Hungary, two collections of isolates were studied: 22 representatives of a collection of 238 MRSA isolates recovered between 1994 and 1998, and a collection of 299 MRSA isolates recovered between 2001 and 2004. The isolates were first characterised by pulsed-field gel electrophoresis (PFGE) and were distributed into 19 different PFGE patterns. Representatives of each pattern were further characterised by *spa* typing, multilocus sequence typing (MLST) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. The Hungarian clone that was predominant in 1994-1998 (PFGE E, ST239-III) had almost disappeared in 2003–2004, being replaced by the Southern German clone (PFGE B, ST228-I) and the New York/ Japan epidemic clone (PFGE A, ST5-II), which represented *c.* 85% of the 2001–2004 isolates. Thus, this study describes, for the first time, the co-dominance and extensive spread of the New York/ Japan clone in a European country.

INTRODUCTION

During the past two decades methicillin-resistant *Staphylococcus aureus* (MRSA) has become the most prevalent and important nosocomial pathogen, causing serious infections such as skin abscesses and wound infections, osteomyelitis, endocarditis, pneumonia, meningitis, bacteraemia and toxic shock syndrome (25). More recently, MRSA infections have been reported in the community among patients with and without traditional risk-factors for MRSA infection. These latter infections are associated predominantly with skin and soft-tissue abscesses and cellulitis (39).

In Europe, the prevalence of MRSA varies widely among nations and is consistently higher in southern countries, e.g., Portugal, Spain, Italy, France and Greece, which report a prevalence of >30%, as compared with <2% in Scandinavian countries, Switzerland and The Netherlands (http://www.earss.rivm.nl). In central and eastern Europe, the prevalence of MRSA has been estimated at *c.* 15% in Austria, the Czech Republic and Slovenia, between 20% and 37% in Hungary, Slovakia, Poland, Bulgaria and Croatia, and >60% in Romania (http://www.earss. rivm.nl) (44).

Epidemiological studies using molecular typing methods have indicated that the massive geographical spread of MRSA results from the dissemination of a few highly epidemic clones. These major epidemic clones belong to sequence type (ST) 5 (SCC*mec* II - New York/ Japan, and SCC*mec* IV - Pediatric), ST239 (SCC*mec* III/IIIA - Brazilian), ST247 (SCC*mec* IA - Iberian), ST22 (SCC*mec* IV - EMRSA-15), ST36 (SCC*mec* II - EMRSA-16) and ST45 (SCC*mec* IV - Berlin) (4, 18, 35).

Another epidemic MRSA clone, sharing ST239 with the Brazilian clone, was described for the first time in Hungary, and was reported to be widely dispersed in Hungarian hospitals between 1993 and 1998 (14, 32). Further studies reported the local predominance of this clone in other regions of the world, namely Taiwan and China (3), and more recently in India (6), Poland (26) and Norway (21). Norway has one of the lowest rates of MRSA in Europe, which indicates the high capacity for dissemination of this particular clone.

Besides the limited number of MRSA clones circulating in Europe, the phenomenon of clonal replacement has been observed; i.e., clones that were disseminated widely during the beginning of the last decade have become less common and have been replaced by other epidemic clones. In Portuguese hospitals, the prevalent Portuguese clone (ST239-III variant) in the mid-1980s was replaced by the Iberian clone (ST247-IA) in 1992–1993, and later, by the Brazilian clone (ST239-III/IIIA) (4). Another example of clone replacement was observed in German hospitals between 1994 and 2002. The Northern German (ST247-I) and the Hannover (ST254-IV) clones, which were prevalent at the beginning of the 1990s, were replaced in 2000 by the Berlin (ST45-IV), the Southern German and the Barnim (ST22-IV) clones, and 1 year later by the Rhine-Hesse (ST5-II) MRSA epidemic clone (43). Also, in the Czech Republic, Melter et al. (30) reported the arrival of the epidemic EMRSA-15 (ST22-IV) clone, replacing the previously prevalent clones in the country, i.e., the Brazilian or ST239-related clones and the Iberian (ST247-IA) clone.

The incidence of MRSA in Hungary is currently *c.* 20% among invasive isolates of *S. aureus* (http://www.earss.rivm.nl). However, since the late 1990s, there have been no molecular data available from Hungary. Therefore, the aims of the present study were, first, to provide an update of the MRSA clonal types circulating in Hungary between 2001 and 2004, and second, to trace the temporal evolution of the epidemic clones in Hungary during the last decade.

MATERIAL and METHODS

Bacteria

The study investigated two collections of isolates: (i) 299 isolates selected from among 3539 MRSA isolates, recovered between 2001 and 2004 from different provincial hospitals located in 18 of the 19 Hungarian counties (Figure 1), sent to the Hungarian National Center for Epidemiology as part of routine MRSA surveillance-the 299 isolates were all single patient invasive isolates (from blood cultures or cerebrospinal fluid), with 15 isolates from 2001, 48 isolates from 2002, 103 isolates from 2003, and 133 isolates from 2004; and (ii) 22 clinical MRSA isolates selected from among 238 MRSA isolates recovered between 1994 and 1998 in six Hungarian counties (Figure 1) (32) - these 22 isolates represented all of the pulsed-field gel electrophoresis (PFGE) profiles described by Oliveira et al. (32), and were included in the present study to enable a more detailed characterisation in order to trace the evolution of the clonal types circulating in Hungary between 1994 and 2004.

Antimicrobial susceptibility testing

Susceptibility to oxacillin, vancomycin, ciprofloxacin, erythromycin, clindamycin, gentamicin, trimethoprim–sulphamethoxazole, tetracycline, rifampicin, teicoplanin, quinupristin–dalfopristin and linezolid was tested by the disk-diffusion method according to CLSI guidelines (9). Antimicrobial susceptibility testing for oxacillin and vancomycin was also performed using Etests (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. *S. aureus* ATCC 25923 was used as a quality control strain for antimicrobial susceptibility testing.

Phage typing

The isolates recovered between 2001 and 2004 were typed using the international basic set of phages for *S. aureus* isolates of human origin, and a set of ten phages selected by Richardson et al. (37) that were developed in certain countries in response to local problems in typing MRSA strains. All phages were used at 100·the routine test dilution.

PFGE

PFGE was performed for all isolates as described by Chung et al. (11). In brief, genomic DNA samples immobilised in agarose were digested overnight with SmaI (New England BioLabs, Beverly, MA, USA), followed by resolution of the restriction fragments in a CHEF-DRIII contour-clamped homogeneous electric field apparatus (Bio-Rad, Hemel Hempstead, UK). PFGE patterns were analysed visually and interpreted using the criteria of McDougal et al. (29), and were also analysed using BioNumerics v.4.0 (Applied Maths, Sint-Mar-tens-Latem, Belgium). Concatamerised phage λ DNA (New England BioLabs) and a SmaI genomic digest of *S. aureus* NCTC 8325 were used as internal markers to normalise the gels. Percentage similarities were determined from a dendrogram that was constructed using the unweighted pair group with arithmetic averages (UPGMA) method and the Dice coefficient, with band position tolerance and optimisation set at 1%. According to Carriço et al. (10), and after reviewing the epidemiological data associated with each of the clusters, a similarity coefficient of 80% was used to define the PFGE type clusters.

***spa* typing**

Analysis of polymorphism in the X region of the protein A gene (*spa*) was performed as described previously (41) for at least one representative of each PFGE type (n = 40). The *spa* types were determined by assignment according to the Ridom web server (http://www.ridom.de/spaserver/), using Ridom StaphType v.1.4.10 software (Ridom GmbH, Wurz-burg, Germany) and the eGenomics web server (http:// tools.egenomics.com/). As observed previously (2, 41), *spa* types with similar repeat profiles were grouped together in spa lineages, which were identified in the present study by numbers.

Multilocus sequence typing (MLST)

MLST was performed on at least one representative of each PFGE pattern (n = 49) by PCR amplification of internal fragments of seven housekeeping genes as described by Enright et al. (19), except that primer *arcC*F2 (5'-

CCTTTATTTGATTCACCAGCG) was used (13), or according to van Leeuwen et al. (45). The allelic profile, and hence the ST, of each isolate was identified using the MLST database (http:// www.mlst.net).

SCC*mec* and *ccrAB* typing

The staphylococcal cassette chromosome *mec* (SCC*mec*) type was determined for at least one representative of each PFGE pattern/*spa* lineage (*n* = 66) using the multiplex PCR strategy developed by Oliveira and de Lencastre (33), which assembles a specific amplification profile for each cassette type. A *ccrAB* (cassette chromosome recombinase)-specific PCR (24) was performed to confirm the presence of SCC*mec* type IV, and also when a non-conclusive result was obtained using the multiplex strategy. Amplification of *ccrB* (34) was also performed for one isolate that was not typeable using the above approaches.

Panton-Valentine leukocidin (PVL) gene analysis

The presence of the PVL *lukS*/*lukF* genes was investigated by PCR (27) for representatives of each PFGE pattern.

RESULTS

Antibiotic profiles and phage typing

The 321 isolates were assigned to 12 antibiotypes (Table 1). All isolates were resistant to oxacillin, and high proportions were resistant to erythromycin (95.6%), ciprofloxacin (95.3%), clindamycin (87.9%) and gentamicin (58.9%). None of the isolates was resistant to linezolid, teicoplanin, quinupristin–dalfopristin or vanco-mycin.

Among the 299 isolates collected between 2001 and 2004, 193 (64.5%) were typeable with both sets of phages: 154 belonged to phage group III, 13 to group 83A, ten to group I, nine to group 95, and seven to group 80. The remaining 106 isolates, which could not be assigned to a defined group, included 58 non-typeable isolates and 48 belonging to mixed groups. These 48 isolates included 22 isolates from mixed groups III + 95, nine isolates from groups III + I, six isolates from groups III + II, and five isolates from groups III + 94/96. Mixed groups I + 83A, I + 94/96 and II + 83A each contained a single isolate, and the remaining three isolates belonged to groups 83A + 94/96.

Macrorestriction analysis

All 321 MRSA isolates were studied by PFGE. Although the isolates were distributed into 19 PFGE patterns (types A-C, E-L, P-T, V, X and Z) differing by six or more bands, two major clonal groups could be distinguished: pattern B, accounting for 141 (44%) of the isolates, and pattern A, accounting for 113 (35.2%) isolates. With the exception of pattern E (*n* = 13, 4.5%), all remaining clones were represented by <4%of the isolates, and were considered to be minor clones (Table 1).

spa typing, MLST and SCC*mec* typing

Representatives of the 19 PFGE patterns were characterised further by *spa* typing (*n* = 40), MLST (*n* = 49) and SCC*mec* type (*n* = 66) (Table 1). According to *spa* typing, the isolates were assigned to seven *spa* lineages. MLST identified 11 different sequence types (ST5, ST228, ST239, ST36, ST254, ST247,

ST8, ST1, ST45, ST22 and ST875), which belonged to seven clonal complexes (CC5, CC8, CC1, CC30, CC45, CC22 and CC12).

All four of the typical SCC*mec* types I-IV, as well as variants IA, IIIA and IVA, were detected among the isolates investigated. One isolate, belonging to CC12, was *mecA*-positive, but was not typeable by the PCR multiplex strategy (33), or by *ccrAB* (24) and *ccrB* (34) typing. In the two predominant clonal complexes, CC5 appeared to be associated with both SCC*mec*I and SCC*mec*II, while SCC*mec* III and SCC*mec* IIIA were detected only in CC8, in association with ST239.

Although PFGE patterns A and B, which included most (*n* = 254) of the isolates, were assigned to a specific *spa* type, i.e., *spa* type t002/2 or *spa* type t041/388 (RIDOM/eGenomics nomenclature), respectively, they shared the same *spa* motif r20r17r12r17r16 (eGenomics nomenclature: DMGMK) and were both associated with *spa* lineage 1. Lineage 2, motif r16r02r25r17r24 (eGenomics nomenclature: KAOMQ), included nine PFGE patterns that, with the exception of pattern L, all belonged to ST239-SCC*mec* type III (or IIIA).

Of the 11 STs identified, two predominated (>113 isolates each) and belonged to CC5: (i) ST5, associated with PFGE patterns A and J, and with SCC*mec*II; and (ii) ST228, a double-locus variant of ST5 in the *tpi* and *yqiL* alleles, corresponding to PFGE pattern B and SCC*mec* I.

PVL screening

Representatives (*n* = 65) of each PFGE pattern were selected randomly and tested for the presence of the PVL determinant. None of the isolates tested positive for the genes encoding PVL.

TABLE 1. Clonal distribution of methicillin-resistant *Staphylococcus aureus* isolates recovered during 1994 – 1998 and 2001 – 2004 in Hungarian hospitals.

Isolate[a]	Year of isolation	PFGE pattern	No. of isolates	Phage group[b]	*spa* lineage	*spa* typing		ST	CC	SCC*mec* type	Antibiotype	Antibiogram											
						Ridom[c]	Kreiswirth[d]					Oxa	Cip	Gen	Van	Tei	Rif	Tet	SXT	Ery	Cli	Lin	Q-D
HU245	2001	A	113	III	1	t002	2	5	5	II	1	R	R	S	S	S	S	S	S	R	R	S	S
HU350	2004	B	141	III	1	t041	388	228	5	I	2	R	R	R	S	S	S	S	S	R	R	S	S
HU152	1994	C	3	NT	2	t019	19	239	8	III	3	R	R	R	S	S	S	R	R	R	R	S	S
HU2	1996	E	13	III	2	t989	389	239	8	III	3	R	R	R	S	S	S	R	R	R	R	S	S
HU290	2002	F	8	Mixed	3	t139	649	254	8	IV variant	4	R	R	R	S	S	S	R	R	R	S	S	S
HU372	2004	G	1	NT	5	t156	755	875	12	NT	5	R	S	S	S	S	S	S	S	S	S	S	S
HU332	2003	H	3	III	6	t127	35	1	1	II	12	R	S	S	S	S	S	R	S	R	S	S	S
HU125	1994	I	1	III	2	t037	3	239	8	III	11	R	S	R	S	S	S	R	S	R	S	S	S
HU363	2004	J	11	III	1	t062	12	5	5	II	2	R	R	R	S	S	S	S	S	R	R	S	S
HU281	2002	K	3	III	4	t038	756	45	45	IV	5	R	S	S	S	S	S	S	S	S	S	S	S
HU275	2002	L	2	NT	2	t018	16	36	30	II	2	R	R	R	S	S	S	S	S	R	R	S	S
HU288	2002	P	3	NT	3	t008	1	8	8	IV	6	R	R	S	S	S	S	S	S	S	S	S	S
HU270	2002	Q	8	NT	2	t030	351	239	8	III	7	R	R	R	S	S	R	R	S	R	R	S	S
HUR4	1997	R	1	ND	2	t787	393	239	8	III	8	R	R	R	S	S	S	R	S	R	S	S	S
HU248	2001	S	4	III	2	t037	3	239	8	III	9	R	R	R	S	S	I	R	I	R	R	S	S
HUR97	1998	T	1	ND	3	t051	4	247	8	IA	10	R	R	R	S	S	R	R	S	S	S	S	S
HU303	2003	V	2	III	7	t032	382	22	22	IV	6	R	R	S	S	S	S	S	S	S	S	S	S
HU107	1996	X	2	III	2	t037	3	239	8	III	12	R	S	S	S	S	S	R	S	R	S	S	S
HU272	2002	Z	1	Mixed	2	t787	393	239	8	III	3	R	R	R	S	S	S	R	R	R	R	S	S

[a] Isolates selected correspond to representatives of each pulsed-field gel electrophoresis (PFGE) pattern.
[b] The isolates are representatives of the major phage group within each PFGE pattern. NT, not typeable; ND, not determined; Mixed, the isolates belong to mixed phage groups.
[c] The nomenclature adopted was that of the RIDOM web server, according to Harmsen et al. (23).
[d] The nomenclature adopted was that of the eGenomics web server, according to Shopsin et al. (41).
ST, sequence type; CC, clonal complex; R, resistance; I, intermediate susceptibility; S, susceptibility; Oxa, oxacillin; Cip, ciprofloxacin; Gen, gentamicin; Van, vancomycin; Tei, teicoplanin; Rif, rifampicin; Tet, tetracycline; SXT, trimethoprim-sulphamethoxazole; Ery, erythromycin; Cli, clindamycin; Lin, linezolid; Q-D, quinupristin-dalfopristin.

Temporal and geographical clonal distribution

The evolution of MRSA clones in Hungarian hospitals between 1994 and 2004 is summarised in Figure 2. Previous data (32) and the results obtained for the 22 representative isolates by *spa* typing, MLST and SCC*mec* typing showed that 99% of the isolates (i.e., all except two isolates) from 1994-1996 belonged to ST239-III. Several PFGE patterns were associated with the ST239-III isolates, but the Hungarian clone (PFGE E) was clearly predominant (74%). Over time, the prevalence of ST239-III clones, including the Hungarian clone, decreased progressively to a minimum of <0.5% in 2003- 2004. In parallel, two other well-represented clones emerged in Hungary: (i) PFGE B-ST228-I (the Southern German clone) was introduced in 1997-1998, represented by 28% of the isolates; and (ii) PFGE A-ST5-II (the New York/ Japan clone) emerged in 2001-2002, with a prevalence of 30%. Over time, the prevalence of both clones increased, reaching 50% and 40%, respectively, in 2003-2004, at which time they became the two most prevalent clones isolated in Hungary. The two isolates recovered in 1994-1996 that did not share ST239 were found to belong to the Hannover clone (PFGE F-ST254-IV). This clone, absent during 1997-1998, re-emerged during 2001-2002 (6%), but remained at a relatively low prevalence (1%) during the following years.

The MRSA isolates from 1994-1998 were recovered from six of the 19 Hungarian counties, while the prevalent Hungarian clone ST239-III was found in five different counties throughout the country (Figure 1). Despite the fact that the county in which the capital was located was over-represented among the 2001-2004 isolates, all except one of the remaining counties were represented in this collection (Figure 1). During this second study period, the Hungarian clone (or ST239-related clones) was still present in four counties throughout Hungary, three of which were included in the 1994-1998 study, as well as in an additional southern county. The Southern German clone ST228-I, which was restricted to one county until 1999, was isolated subsequently in nine additional counties throughout Hungary. Moreover, the New York/ Japan clone ST5-II, present in Hungary only since 2001, was widespread throughout 11 counties. The rapid dissemination of these clones might be linked to the transfer of Hungarian patients from hospital to hospital, and hence from county to county.

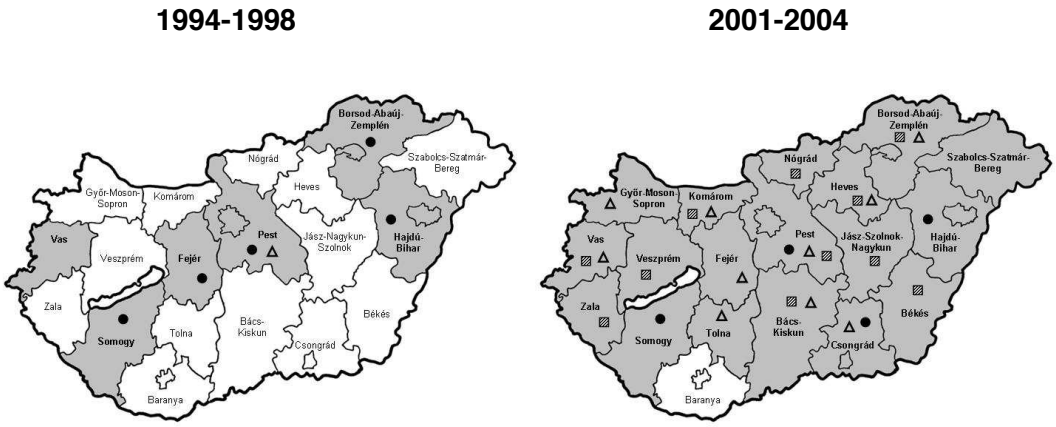

Figure 1. Comparison of the geographical distribution of the major methicillin-resistant *Staphylococcus aureus* clones in Hungary during 1994-1998 and 2001-2004. The Hungarian clone (ST239-III) is represented by circles, the Southern German clone (ST228-I) by triangles, and the New York/ Japan clone (ST5-II) by squares. The counties studied in each period are highlighted in grey.

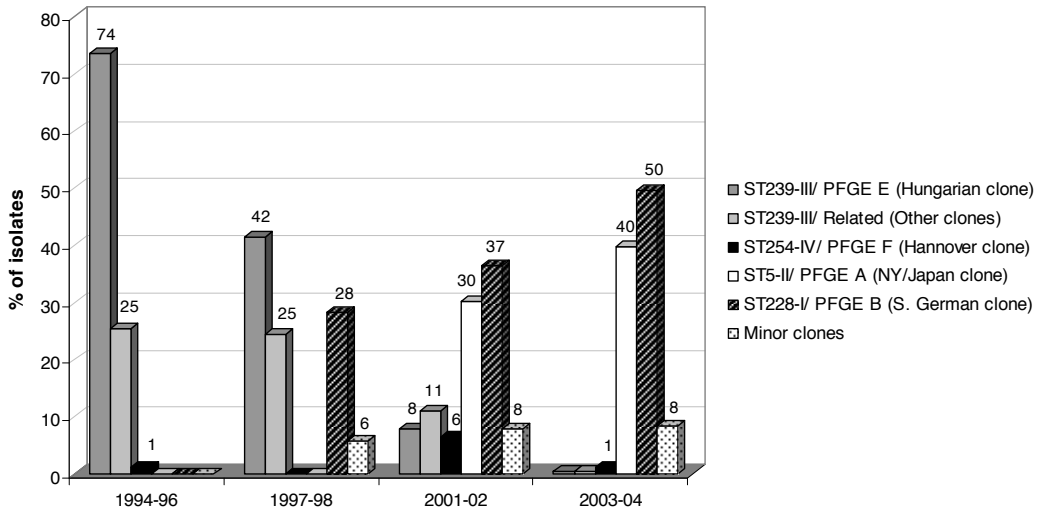

Figure 2. Clonal evolution of methicillin-resistant *Staphylococcus aureus* over a 10-year period in Hungarian hospitals (numbers over each bar indicate the number of isolates).

DISCUSSION

Few surveillance studies have described the evolution of MRSA in central and eastern Europe. Although there was a gap in the present study between 1999 and 2000, during which MRSA isolates from Hungary were not obtained, this report describes the evolution of the clonal types circulating in Hungary over the last decade.

The predominance of a single clone in Hungarian hospitals between 1993 and 1994 was reported by de Lencastre et al. (14). This Hungarian clone, characterised by ST239-III, was related closely to the epidemic Brazilian clone, which is dispersed widely in Europe, South America and Asia (4). Oliveira et al. (32) used a combination of *spa* typing and PFGE typing to reveal a significant decrease in the prevalence of the Hungarian clone, from c. 74% in 1994-1996 to 42% in 1997-1998. Although the Hungarian clone has also been described in Poland (26) and Norway (21), the present results show that it is currently almost non-existent in Hungary. Two other clones, both belonging to CC5, i.e., the Southern German (ST228-I) and the New York/ Japan (ST5-II) clones, are now the predominant clones in Hungarian hospitals.

The Southern German clone was first described in the southern area of Germany in 1992, and spread throughout Germany during 1995-1996 (48). About 1 year later, this clone was detected in Budapest, the Hungarian capital, with a prevalence of 28%, and then spread rapidly throughout Hungary, becoming the prevalent clone in 2003-2004. ST228-I has also been reported to be the predominant clone (86% of MRSA isolates) in a hospital in Slovenia (31). In Croatia, which also borders Hungary, Budimir et al. (8) reported that ST111-I, a single-locus variant of ST228 with the same SCC*mec* type, occurred at a prevalence of 52% in bloodstream isolates. Moreover, other minor clones associated with ST228-I have been reported in Italy (28), Denmark (20), Belgium (16) and Switzerland (40) (http://www.mlst.net), showing that clone ST228-I, or related STs, are relatively common in Europe.

After being isolated in northern European countries (4), ST5 was detected in Hungary soon after its double-locus variant, ST228. ST228 seems to have

evolved from ST5 (38). However, the appearance of these two clones in Hungary probably represents independent events, since ST228 was the first to be detected (Figure 2). ST5 appeared almost simultaneously in 11 counties, whereas ST228 was first isolated in the restricted area of Budapest, and only spread later throughout the country. Interestingly, to our knowledge, the New York/ Japan clone has never been described in countries bordering Hungary, and was therefore probably imported from countries in which it is highly prevalent, i.e., the USA, Japan, Korea and Mexico (5, 17, 42). Nevertheless, clone ST5-II has been reported recently in some Belgium hospitals, as was the ST45-IV clone, which was predominant among 251 isolates recovered from 95 hospitals during 2003 (15). ST45-IV was also isolated in Hungarian hospitals, but with a very low prevalence (three isolates). Other epidemic clones have been described in central Europe, e.g., EMRSA-15 (ST22-IV), which has been detected in hospitals throughout all regions of the Czech Republic, replacing both the Brazilian (ST239) and related clones, and the Iberian clone (ST247) (30). Interestingly, one of the minor clones described in Hungary, PFGE pattern V (Table 1), belonged to ST22-IV and was represented by two isolates from a single county. Thus, ST5 and related clones, such as ST228 and ST111, as well as the non-multiresistant EMRSA-15 (ST22) clone, are becoming the major clones circulating in eastern European countries, in addition to the Iberian and the Brazilian or ST239-related clones.

Concerning the 2001-2004 MRSA collection, with the exception of phage groups 80 and 83A, which belonged only to PFGE pattern A, the other phage groups (I and III) included more than one PFGE pattern. In the two major PFGE patterns, A and B, most isolates belonged to phage group III. Phage typing is still used to characterise *S. aureus* (47), but this collection showed low typeability, with 35.4%of the isolates not being assigned to any group (i.e., belonging to mixed phage groups or not typeable). There was poor concordance between phage typing and the DNA-based approaches used, as has been observed previously (7).

Although the PVL determinant, a bicomponent leukotoxin virulence factor linked to severe necrotising fasciitis and necrotising pneumonia, has been detected previously in the hospital setting (1, 36), it is associated more commonly

with community-acquired MRSA strains harbouring SCC*mec* types IV or V (22); it was not detected in any of the nosocomial Hungarian isolates in the present study. Voyich et al. (46) recently reported that strains lacking PVL were as virulent in mouse sepsis and abscess models as those that produce the leukotoxin, and therefore concluded that PVL is not the major virulence determinant of community-acquired MRSA.

In summary, the present results indicate that a temporal recycling of the prevalent clones circulating in Hungary occurred between 1994 and 2004. Although ST239-related clones are still isolated in central Europe, e.g., in Poland (26) and Romania (12), the prevalence of ST239-related clones is currently almost null in Hungary. A progressive replacement of the Hungarian clone by the Southern German and New York/ Japan epidemic clones was observed in the hospital setting between 2001 and 2004, and the co-dominance and massive spread of the New York/ Japan clone in a European country was observed for the first time.

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T. Conceição performed the experimental work, analyzed the results and wrote the manuscript.

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Chapter VII

Staphylococcal interspersed repeat unit typing of *Staphylococcus aureus*: evaluation of a new multilocus variable-number tandem-repeat analysis typing method

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ABSTRACT

The present study evaluates the performance of the staphylococcal interspersed repeat unit (SIRU) method applied to a diverse collection of 104 *Staphylococcus aureus* isolates previously characterized by pulsed-field gel electrophoresis (PFGE), *spa* typing, multilocus sequence typing (MLST), and staphylococcal cassette chromosome *mec* typing for methicillin-resistant *S. aureus*. The SIRU method distributed the 104 strains into 81 SIRU profiles that could be clustered into 12 groups and 29 singletons. The discriminatory power of the method at the profile level, translated by Simpson's index of diversity (SID), was similar to that of PFGE subtyping (SID = 99.23% versus 99.85%) and slightly higher than that of *spa* typing (SID = 97.61%). At the group level, the SIRU SID (93.24%) was lower than that of PFGE typing (95.41%) but higher than that of MLST (SID = 91.77%). The adjusted Rand (AR) coefficient showed that SIRU typing at the group level had the highest congruence with MLST (AR = 0.5736) and with clonal complex (CC) (AR = 0.4963) but the lowest congruence with PFGE subtype (AR = 0.0242). The Wallace coefficient indicated that in the present collection, two strains with the same SIRU profile have a 100% probability of belonging to the same CC, a 90% probability of sharing the same *spa* type, and an 83% probability of being classified in the same sequence type. The high discriminatory power of the SIRU method, along with its apparent concordance with MLST results, makes it potentially valuable for *S. aureus* short-term epidemiological investigations and population dynamics as well.

INTRODUCTION

Staphylococcus aureus, especially methicillin-resistant *S. aureus* (MRSA), continues to be a major cause of health care-associated and, more recently, community-associated infections (40, 62). It is critical to have access to an accurate typing method to design cost-effective intervention and prevention strategies (45, 48, 61). A large number of molecular typing methods have been developed to assess strain relatedness for outbreak control, surveillance programs, and population structure and evolution studies (58, 61). The three most used typing methods for *S. aureus* have advantages and disadvantages, as follows: (i) Pulsed-field gel electrophoresis (PFGE), which is the "gold standard" typing method, has high discriminatory power and accuracy, but it is time-consuming and expensive, and the interlaboratory exchange of results is challenging; (ii) Sequence-based multilocus sequence typing (MLST) is easy to perform, and the results, given as an allelic profile, are portable and easy to exchange due to a public database available on the Internet (http://www.mlst.net), but it is expensive and not useful for local outbreak investigations. MLST is frequently combined with staphylococcal cassette chromosome *mec* (SCC*mec*) typing in order to define clonal types of MRSA (24); (iii) *spa* typing, a single-locus sequence typing method, is being used more frequently for *S. aureus* typing, and the development of a public database on the Internet (http://spaserver.ridom.de), as with MLST, ensured an international typing nomenclature and thus a great facility in exchanging typing data. By calculation of Simpson's index of diversity (SID), it was shown that *spa* typing is nearly as discriminatory as PFGE (1, 25), although it takes into account a single variable region of the protein A gene.

In choosing a new method, it is worth taking into consideration that PCR-based methods are commonly used in typing laboratories because of their accuracy, ease of use, low cost, and speed in retrieving results (in a few hours).

Many bacterial genomes carry loci of repetitive DNA, which may contain variable repeated units among strains (43, 60). Systems based on a multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) have been used extensively for typing of clinical isolates of several bacterial species and were

shown to perform well compared to other genotyping methods (43, 61). *S. aureus* harbors a diverse population of DNA repeats, which allowed the design of various MLVA schemes (28, 30, 39, 54, 60). Hardy et al. (36, 38) developed a MLVA scheme for *S. aureus* where seven novel multiple tandem repeats with a high degree of similarity in the flanking regions were identified based on the alignment of seven *S. aureus* sequenced genomes (strains N315, MW2, Mu50, MSSA476, MRSA252, NCTC8325, and COL). Six of these seven loci were located on intergenic regions scattered around the *S. aureus* genome; the remaining locus corresponds to the protein A gene, *spa*. The method, designated staphylococcal interspersed repeat unit (SIRU) typing, relies on PCR amplification of the seven loci of repetitive DNA, using primers specific for the flanking regions of each locus, and on the determination of the size of each amplicon, which reflects the number of repeated units present on the targeted SIRU. To each of the seven loci is attributed the respective number of DNA repeats, generating a combination of seven numbers that characterizes each strain and corresponds to the allelic profile. This allelic profile makes the SIRU method amenable to interlaboratory comparisons and database management, comparable to MLST. So far, the SIRU method has been applied to *S. aureus* isolates from nosocomial outbreaks in the United Kingdom and Germany, mainly MRSA isolates, and therefore to highly related strains (29, 35-37). Very recently, a single study evaluated a MLVA scheme including the SIRU typing loci and the *sspA* gene, using a European collection of contemporary *S. aureus* isolates (39).

The aim of the present study was to evaluate the SIRU method with a more diverse collection of *S. aureus* isolates, including MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates, from different continents, isolated throughout several decades and previously characterized by well-established typing methods (PFGE, *spa* typing, MLST, and SCC*mec* for MRSA).

MATERIAL and METHODS

Bacterial isolates

A collection of 104 strains (78 MRSA and 26 MSSA strains), previously characterized by PFGE (16), *spa* typing (1, 56), MLST (9), and SCC*mec* typing (for MRSA strains) (46), was selected from the Laboratory of Molecular Genetics collection at Instituto de Tecnologia Química e Biológica, Oeiras, Portugal (Table 1). The selected collection included hospital- and community-related strains isolated during a period of over 50 years (from 1943 to 2006) from 17 countries distributed over four continents. Efforts were made to select strains with various degrees of genetic relatedness. Therefore, the collection included (i) strains belonging to the five main clonal complexes (CCs) of *S. aureus* - CC5 (*n* = 23), CC8 (*n* = 32), CC22 (*n* = 4), CC30 (*n* = 7), and CC45 (*n* = 9); (ii) isolates belonging to minor CCs - CC1 (*n* = 7), CC509 (*n* = 3), CC50, CC59, CC97, CC101, CC228, CC398, and CC1021 (one isolate of each); (iii) two isolates belonging to CC80, ST80, and SCC*mec* type IV, identified as community-acquired MRSA; (iv) six single CC isolates, referred to as singletons (S1, sequence type 157 [ST157]; S2, ST447; S3, ST668; S4, ST707; S5, ST580; S6, ST445); and (v) four strains belonging to nondefined CC groups (CC assignments assessed by eBURST v3) and designated ND1 to ND4.

Strains N315, NCTC8325, COL, and MW2 were included for reproducibility and methodology control, since their genomes are fully sequenced and were used in the theoretical design of the SIRU method (38).

PFGE analysis

PFGE patterns were analyzed in BioNumerics, version 4.61, software (Applied Maths, Sint-Martens-Latem, Belgium) as previously described (25), with minor modifications, including an optimization setting of 1.0% for band pattern comparisons and a 98% Dice coefficient similarity cutoff for PFGE subtypes.

spa typing and MLST analysis

spa types were assigned through the Ridom web server (http://spaserver.ridom.de). Additionally, for one isolate previously characterized as nontypeable, the *spa* type was determined through sequencing of SIRU21 (see below). MLST alleles and STs were identified through the MLST database (http://www.mlst.net), and CCs were defined using the eBURST v3 algorithm (http://eburst.mlst.net).

SIRU typing

DNA was extracted as previously reported (9). The SIRU method was performed as previously described (36, 38), with the following minor modifications: (i) 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems, CA) was used per PCR in a final volume of 25 µl, (ii) an annealing temperature of 59°C was used with primer SIRU05R2 (see below), and (iii) PCR products (10 µl) were resolved in a 2.5% Seakem LE (Cambrex, Rockland, ME) agarose gel in 0.5% Tris-borate-EDTA buffer (Bio-Rad, Hercules, CA) at 5 V/cm for 2.5 h. The size of each amplicon was determined by visual inspection by comparison with a 50-bp ladder size marker and by computer analysis using Bionumerics, version 4.61, software, which facilitates marker-based normalization of the migration distances and therefore guarantees accurate length measurements. The number of repeats was calculated, taking into account the combined size of the repeat unit and the flanking regions of each locus, as follows: (i) for SIRU01, repeat unit of 55 bp + flanking regions of 184 bp = 239 bp for one-repeat-length amplicon; (ii) for SIRU05, 60 bp + 146 bp = 206 bp; for SIRU05 with primer SIRU05R2, 60 bp + 156 bp = 216 bp; (iii) for SIRU07, 56 bp + 191 bp = 247 bp; (iv) for SIRU13, 64 bp + 148 bp = 212 bp; (v) for SIRU15 131 bp + 212 bp = 343 bp; (vi) for SIRU16, 159 bp + 162 bp = 321 bp; and (vii) for SIRU21, 24 bp + 96 bp = 120 bp.

The primers used to amplify each of the seven loci were previously published (36), except for those for locus 16 (SIRU16) and an additional reverse primer for locus 5 (SIRU05). New SIRU16 primers, SIRU16_2F (5'-TGGTGTTAATTTAGCTTGC-3') and SIRU16_2R (5'-AAACGCAACTTGAAGAAACG-3'), were designed through sequence alignments

of the SIRU16 loci of the seven *S. aureus* genomes previously considered for the design of the primers for the remaining loci (38). The new SIRU05 locus reverse primer was designed specifically for strains for which there was no amplification with the previously published primers (SIRU05L and SIRU05R), namely, for strains belonging to STs 1, 22, 45, and 80. Therefore, primer SIRU05R2 (5'-AGTTGTAGTCATCTTACTGC-3') was designed through sequence alignments of the available SIRU05 loci of MW2, MSSA476 (both ST1), and EMRSA-15 (ST22) (sequence from the EMRSA-15 genome sequencing project at the Wellcome Trust Sanger Institute [http://www.sanger.ac.uk/sequencing/Staphylococcus/aureus/EMRSA15]). All sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), with default parameters.

eBURST v3 software was used to cluster SIRU profiles. Isolates sharing six of seven loci with at least one isolate (single-locus variants) were included in the same SIRU group. Singletons represent profiles that appear once and have no related profiles in the collection.

Comparison of typing methods

SID and the respective confidence intervals were calculated as described previously (33, 57). The quantitative level of congruence between typing methods was assessed by a framework proposed by Carriço et al. (15), based on the adjusted Rand (AR) and Wallace (W) coefficients, available at http://www.comparingpartitions.info/. The AR coefficient quantifies the global agreement between two methods, whereas the W coefficient indicates the probability that two isolates classified as the same type by one method are also classified as the same type by another method (15).

RESULTS

SIRU typeability

In a first approach, we tested the method with four completely sequenced strains (N315 [ST5-SCC*mec* II], NCTC8325 [ST8-MSSA], COL [ST250-SCC*mec* I], and MW2 [ST1-SCC*mec* IV]) for which theoretical SIRU profiles had previously been published (36, 38). Five of the seven loci were amplified from the four tested strains; SIRU16 was amplified from strain MW2 only, whereas SIRU05 was amplified from all strains except MW2. A BLAST search showed that the published SIRU16 forward primer (SIRU16_L) (36) has similarity with strain MW2 but not with the three remaining tested strains. The SIRU16_L primer was found to have similarity with strain MSSA476, which belongs to ST1, like MW2, and with strain MRSA252 (ST36-SCC*mec* II). The primer was tested on additional ST36 isolates, and it performed well. These preliminary results led us to design a new SIRU16 primer that anneals with all available genomes of *S. aureus* strains (see Materials and Methods). The SIRU typing method using the new SIRU16 primers was applied to the whole collection. A seven digit profile was obtained for only 70 of 104 strains, indicating a typeability of 67% when isolates with at least one nonamplified locus were considered nontypeable.

Considering the typeability of each locus separately, SIRU07 was the unique locus that showed 100% typeability. SIRU05 showed the lowest typeability (72%), followed by SIRU15 (95%), while the remaining SIRUs showed a typeability of 99% (one isolate was nontypeable). The consistent nonamplification of SIRU05 from all strains belonging to particular CCs, i.e., CC22, CC45, and CC80, led us to perform a BLAST search with different *S. aureus* genomes which showed that the published SIRU05 reverse primer (SIRU05_R) (36) has no similarity with strains MW2, MSSA476, EMRSA-15 (ST22), and RF122 (ST151). Therefore, a new SIRU05R2 reverse primer was designed considering these particular strains (see Materials and Methods), and a new PCR was performed on all isolates for which this locus was not amplified with the previously published SIRU05R primer.

The sequential use of the SIRU05R2 reverse primer increased the typeability from 67% to 89.4%. Moreover, the typeability of the SIRU05 locus alone

rose to 95%. Among the 11 nontypeable isolates, 9 isolates showed no amplification for a single locus, 1 isolate showed no amplification for two loci (SIRU05 and SIRU15), and another isolate showed no amplification for three loci (SIRUs 1, 15, and 16).

For further evaluation of the method, an arbitrarily chosen neutral number of repeats (99) was attributed to all non-amplified loci. However, this artifact creates a limitation because two isolates showing no amplification at the same locus are considered identical in this allele.

SIRU locus discriminatory power

Considering the discriminatory power of each locus, SIRU07 showed four different allele numbers, SIRU16 showed five, SIRU01 and SIRU13 showed six, and SIRU15 showed eight. Loci 5 and 21 showed the highest variability among the seven loci, with 14 and 13 different allele numbers, respectively. The SIRU21 locus, the only one located in a coding region, the *spa* gene, had a direct correlation with the *spa* type. However, an identical number of repeat units may contain sequence variations which are not detected by SIRU typing.

SIRU typing clonal assignment

The 104 strains were distributed into 81 SIRU profiles (differing in at least one of the seven loci) that could be clustered into 12 groups and 29 singletons (Table 1). Figure 1 shows the assignment of groups when the eBURST v3 algorithm was applied to the collection. The largest SIRU group, G2 (n = 20), included the majority (19 of 23) of the strains belonging to CC5 (ST5, ST85, and ST100) and the related strain CC228-ST228. SIRU group G9 included the nine strains belonging to CC45 (ST45 and ST508). Similarly, G7 included the four CC22 strains (ST22 and ST79), and G10 included the two CC80-ST80 strains. SIRU group G8 included only three (ST30 and ST36) of the seven CC30 strains. The remaining four strains (ST30 and ST34) were classified as singletons. CC8 strains were divided into four SIRU groups (G3, G4, G5, and G6) and four singletons. Curiously, ST247 and ST250 strains were found in G5 only, while

Table 1. Characteristics of the 104 *S. aureus* isolates and SIRU typing results

Strain	Isolation date (yr)	Country	SIRU01	SIRU13	SIRU15	SIRU16	SIRU21	SIRU05	SIRU07	SIRU profile[b]	SIRU group[c]	PFGE type[d]	*spa* type	ST	CC[e]	SCC*mec* type	Reference (s)
IPOP38	2001	Portugal	1	1	1	3	7	5[g]	3	1	G1	T2	t127	1	1	MSSA	(4)
CV73	1997	Cape Verde	1	1	2	3	7	5[g]	3	2	G1	T1	t127	1	1	MSSA	(11)
HU332	2003	Hungary	1	1	2	3	7	5[g]	3	2	G1	F11	t127	1	1	II	(17)
HGSA240	2003	Portugal	1	1	2	3	7	2	3	3	G1	T7	t127	81	1	MSSA	(13)
HSA49	1993	Portugal	2	2	1	3	10	3	1	4	G2	p1	t002	5	5	IV	(6)
122MEXU	1998	Mexico	2	3	1	3	10	3	1	5	G2	M2	t002	5	5	II	(63)
78MEXC	1997	Mexico	2	3	1	3	10	3	1	5	G2	A2	t002	5	5	II	(63)
79MEXC	1997	Mexico	2	3	1	3	10	3	1	5	G2	N3	t002	5	5	II	(63)
ARG229	1995	Argentina	2	3	1	3	10	3	1	5	G2	A4	t002	100	5	II	(6, 8, 18)
BK2464	1990	United States	2	3	1	3	10	3	1	5	G2	A1	t002	5	5	II	(52)
BM18	1989	United States	2	3	1	3	10	3	1	5	G2	B2	t002	5	5	IV	(22, 51)
N315	1982	Japan	2	3	1	3	10	3	1	5	G2	Q2	t002	5	5	II	(41)
47MEXU	1997	Mexico	2	3	1	3	8	3	1	6	G2	A3	t895	5	5	II	(63)
COB3	1996	Colombia	2	3	1	3	7	3	1	7	G2	B3	t045	5	5	IV	(31)
HU245	2001	Hungary	2	3	1	3	10	5	1	8	G2	A1	t002	5	5	II	(17)
HU317	2003	Hungary	2	3	1	3	6	3	1	9	G2	T5	t062	5	5	II	(17)
HU363	2004	Hungary	2	3	1	3	6	3	1	9	G2	T6	t062	5	5	II	(17)
JP1	1997	Japan	2	3	1	3	10	1	1	10	G2	M1	t002	5	5	II	(7)
JP26	1997	Japan	2	3	1	2	10	2	1	11	G2	ZF	t002	5	5	II	(6, 7)
PL72	1991	Poland	2	3	1	3	10	7	1	12	G2	P2	t053	5	5	IV	(42)
POL3	1992	Poland	2	3	1	3	10	8	1	13	G2	B4	t053	5	5	IV	(42)
ARG33	1996	Argentina	2	3	1	3	10	99	1	14	G2	Q1	t001	85	5	IIIA	(6, 8, 18)
ARG64	1996	Argentina	2	3	1	3	10	99	1	14	G2	S1	t001	85	5	I	(6, 8, 18)
HAR41	1998	Germany	2	3	1	3	10	2	1	15	G2	I2	t001	228	228	I	(47)

Table 1. *Continuation.*

Strain	Isolation date (yr)	Country	SIRU01	SIRU13	SIRU15	SIRU16	SIRU21	SIRU05	SIRU07	SIRU profile[b]	SIRU group[c]	PFGE type[d]	*spa* type	ST	CC[e]	SCC*mec* type	Reference (s)
HU2	1996	Hungary	3	0	1	3	12	2	2	16	G3	D2	t989	239	8	III	(23)
HUSA304	1993	United States	3	0	1	3	12	2	2	16	G3	D1	t1053	239	8	III	(23)
HU270	2002	Hungary	3	0	1	2	6	2	2	17	G3	E4	t030	239	8	III	(17)
HU294	2003	Hungary	3	0	1	3	13	2	2	18	G3	D5	t538	239	8	IIIA	(17)
TUR1	1996	Turkey	3	0	1	3	6	2	2	19	G3	E7	t030	239	8	III	(6)
TUR27	1996	Turkey	3	0	1	3	6	2	2	19	G3	E8	t030	239	8	III	(6)
PL46	1995	Poland	4	0	1	2	6	2	2	20	G3	E6	t030	157	S1	III	(42)
CPS22	1985	Portugal	4	0	1	3	6	1	2	21	G4	E1	t421	239	8	IIIvar	(21)
CSP68	1985	Portugal	4	0	1	3	6	1	2	21	G4	E3	t421	239	8	IIIvar	(21)
HSA10	1992	Portugal	4	0	1	3	6	1	2	21	G4	E2	t421	239	8	IIIvar	(6)
GRE108	1998	Greece	4	0	1	3	7	2	2	22	G4	C5	t461	239	8	III	(3, 6)
HGSA142	2003	Portugal	4	0	1	3	7	0	2	23	G4	D3	t037	239	8	IIIA	(13)
HGSA339	2003	Portugal	4	0	1	3	7	0	2	23	G4	C3	t037	239	8	IIIA	(13)
HGSA57	1995	Portugal	4	0	1	3	7	0	2	23	G4	C6	t037	239	8	IIIA	(12)
HSJ216	1997	Portugal	4	0	1	3	7	99	2	24	G4	C1	t037	239	8	IIIA	(10)
HU25	1993	Brazil	4	0	1	3	6	0	2	25	G4	C1	t138	239	8	IIIA	(59)
HU272	2002	Hungary	4	0	1	3	10	2	2	26	G4	R2	t787	239	8	III	(17)
TAW166	1998	Taiwan	4	0	1	3	10	8	2	27	G4	N2	t036	254	8	IV	(5, 6)
TAW97	1998	Taiwan	4	0	1	3	7	3	2	28	G4	C3	t037	239	8	IIIA	(5, 6)
BK1953	1995	United States	4	0	1	4	11	4	2	29	G5	F1	t051	247	8	IA	(52)
HGSA13	1998	Portugal	4	0	1	4	11	4	2	29	G5	N1	t051	247	8	IA	(12)
HUR97	1998	Hungary	4	0	1	4	11	4	2	29	G5	F8	t051	247	8	IA	(50)
COL	1965	United Kingdom	4	0	1	4	10	6	2	30	G5	F6	t008	250	8	I	(51)
E2213	1965	Denmark	4	0	1	4	11	6	2	31	G5	F4	t051	247	8	I	(19)

Table 1. *Continuation.*

Strain	Isolation date (yr)	Country	SIRU01	SIRU13	SIRU15	SIRU16	SIRU21	SIRU05	SIRU07	SIRU profile[b]	SIRU group[c]	PFGE type[d]	*spa* type	ST	CC[e]	SCC*mec* type	Reference (s)
E2453	1965	Denmark	4	0	1	4	11	6	2	31	G5	F2	t051	247	8	I	(19)
HPV107	1992	Portugal	4	0	1	3	11	4	2	32	G5	F1	t051	247	8	IA	(55)
GRE18	1998	Greece	4	0	99	2	7	2	2	33	G6	F5	t037	239	8	III	(6)
HGSA15	1994	Portugal	4	0	99	2	7	99	2	34	G6	C4	t037	239	8	IIIA	(12)
HAR22	1991	United Kingdom	2	3	0	3	15	12[g]	2	35	G7	J1	t022	22	22	IV	(47)
HGSA128	2000	Portugal	2	3	0	3	16	12[g]	2	36	G7	J2	t032	79	22	IV	(6, 12)
HU303	2003	Hungary	2	3	0	3	16	12[g]	2	36	G7	J3	t032	22	22	IV	(17)
IPOP2	2001	Portugal	2	3	99	3	16	12[g]	2	37	G7	J1	t032	22	22	IV	(6)
DEN4415	2001	Denmark	2	1	2	2	9	3	2	38	G8	K1	t021	36	30	II	(26)
HAR24	1993	United Kingdom	2	1	2	2	11	2	2	39	G8	K1	t018	36	30	II	(47)
HGSA202	2003	Portugal	2	1	2	2	11	3	2	40	G8	K3	t018	30	30	MSSA	(13)
DEN4358	2001	Denmark	0	0	0	3	10	1[g]	2	41	G9	H8	t116	45	45	V	(26)
PLN49	1997	Poland	0	0	0	3	10	1[g]	2	41	G9	H9	t015	45	45	IV	(6, 42)
CA04	1998	United States	0	0	1	3	8	1[g]	2	42	G9	H6	t124	45	45	IV	(20)
CV81	1997	Cape Verde	0	0	1	3	11	1[g]	2	43	G9	H3	t861	508	45	MSSA	(11)
HAR38	1996	Germany	0	0	1	3	9	1[g]	2	44	G9	H1	t004	45	45	IV	(47)
HU281	2002	Hungary	0	0	1	3	9	1[g]	2	44	G9	H5	t038	45	45	IV	(17)
HSA19	1992	Portugal	0	0	1	3	7	1[g]	2	45	G9	H4	t1072	45	45	MSSA	(4)
IPO516	2006	Portugal	0	0	1	3	10	1[g]	2	46	G9	H7	t2429	45	45	V	(2)
IPOP56	2001	Portugal	0	0	1	3	10	1[g]	2	46	G9	H2	t1538	45	45	MSSA	(4)
HU109	1996	Hungary	1	0	1	3	8	1	2	47	G9	E5	t982	239	8	III	(50)
GRE14	1998	Greece	4	2	6	3	7	1[g]	1	48	G10	L1	t044	80	80	IV	(3, 6)
HFF189	2005	Portugal	4	2	6	3	7	1[g]	1	48	G10	L2	t044	80	80	IV	This study
CHL5	1997	Chile	2	0	1	3	3	2	1	49	G11	I1	t535	83	5	I	(6, 8)

Table 1. *Continuation.*

Strain	Isolation date (yr)	Country	SIRU01	SIRU13	SIRU15	SIRU16	SIRU21	SIRU05	SIRU07	SIRU profile[b]	SIRU group[c]	PFGE type[d]	*spa* type	ST	CC[e]	SCC*mec* type	Reference (s)
JP82	1997	Japan	2	0	1	3	8	2[g]	1	50	G11	G2	t375	92	509	IVA	(6, 7)
COB111	1998	Colombia	2	1	2	3	7	3	1	51	G12	G1	t1572	84	509	IV	(6, 31)
HFF202	2005	Portugal	2	1	2	3	7	2	1	52	G12	X	t1537	707	S4	MSSA	This study
MW2	1998	United States	1	2	2	3	8	5[g]	3	53	S1	T3	t128	1	1	IV	(14)
IPOP58	2001	Portugal	1	2	3	3	6	2	3	54	S2	U1	t189	188	1	MSSA	(4)
HSJ109	1995	Portugal	4	2	4	2	11	9	3	55	S3	ZC	t1897	573	1	MSSA	(4)
HDE1	1996	Portugal	2	3	99	3	9	12	1	56	S4	B1	t311	5	5	IV	(53)
HBA3	2006	Portugal	2	3	1	2	3	3	1	57	S5	A1	t535	5	5	IV	(2)
HDE288	1996	Portugal	2	3	1	3	9	11	1	58	S6	B1	t311	5	5	IV	(53)
NCTC8325	1943	United Kingdom	3	0	1	4	10	6	3	59	S7	F3	t211	8	8	MSSA	http://www.nctc.org.uk
ARG199	1996	Argentina	4	99	7	3	11	9	1	60	S8	D4	t148	86	8	II	(6, 8, 18)
GRE4	1998	Greece	99	0	99	99	7	3	2	61	S9	R1	t037	239	8	IIIA	(6)
HAR36	1993	Germany	4	0	1	3	13	6	2	62	S10	F7	t009	254	8	IV	(47)
DEN2946	2001	Denmark	2	1	1	2	7	5	2	63	S11	K4	t975	30	30	IV	(26)
CV11	1997	Cape Verde	2	1	2	2	7	4	2	64	S12	K2	t942	30	30	MSSA	(11)
HGSA256	2003	Portugal	2	1	2	2	12	1	1	65	S13	K5	t166	34	30	MSSA	(13)
IPOP24	2001	Portugal	2	1	2	2	5	3	1	66	S14	K6	t1076	34	30	MSSA	(4)
E1114	1960	Denmark	0	1	1	3	8	2	1	67	S15	W	t518	50	50	MSSA	(32)
TAW214	1998	Taiwan	2	1	4	2	7	11[g]	4	68	S16	ZG	t437	59	59	IV	(5, 6)
IPOP50	2001	Portugal	2	2	1	3	9	1[g]	3	69	S17	T4	t359	97	97	MSSA	(4)
IPOP51	2001	Portugal	2	4	2	2	9	99	2	70	S18	U2	t1075	106	101	MSSA	(4)
CV55	1997	Cape Verde	3	1	1	3	8	2	2	71	S19	ZB	t937	291	398	MSSA	(11)
JP87	1997	Japan	2	1	1	3	8	9[g]	1	72	S20	V	t375	89	509	IIvar	(6, 7)
E260	1957	Denmark	2	1	1	0	10	2	3	73	S21	ZE	t1194	446	1021	MSSA	(32)

Table 1. *Continuation.*

Strain	Isolation date (yr)	Country	SIRU01	SIRU13	SIRU15	SIRU16	SIRU21	SIRU05	SIRU07	SIRU profile[b]	SIRU group[c]	PFGE type[d]	*spa* type	ST	CC[e]	SCC*mec* type	Reference (s)
E3373	1967	Denmark	3	1	4	3	8	10	2	74	S22	F10	t164	447	S2	MSSA	(32)
CV87	1997	Cape Verde	3	2	2	2	99	9	4	75	S23	S2	t941	668	S3	MSSA	(11)
DCC1185	1997	Portugal	2	2	2	3	7	2	2	76	S24	ZA	t1065	580	S5	MSSA	(4)
E216	1957	Denmark	1	2	7	2	11	11	1	77	S25	ZH	t1191	445	S6	MSSA	(32)
E691	1959	Denmark	2	1	1	4	13	2	2	78	S26	ZD	t1207	49	ND1	MSSA	(32)
DCC300	1996	Portugal	2	1	1	2	12	2	1	79	S27	Y	t166	10	ND2	MSSA	(4)
DEN2230	2001	Denmark	3	0	2	3	10	2	2	80	S28	Z	t355	152	ND3	V	(26)
CV161	1997	Cape Verde	3	4	2	3	9	1[g]	3	81	S29	F9	t359	669	ND4	MSSA	(11)

[a] 0, the size of the amplicon obtained was shorter than that of a complete repeat; 99, no amplification was obtained.
[b] Numerical nomenclature of SIRU profiles. New numbers are attributed to profiles that differ in at least one allele.
[c] S, singletons, i.e., SIRU profiles that appear once and have no related profiles.
[d] PFGE types and subtypes were determined using thresholds of 80% and 98% similarity, respectively.
[e] CC assignments were assessed by eBURST v3 on 9 June 2008. S, singletons; ND, not determined. The sequence types are included in groups for which no founder could be determined.
[f] Strains that do not have SCC*mec* are labeled as MSSA strains.
[g] SIRU05 amplification using the SIRU05R2 reverse primer.

ST239 strains were distributed over G3, G4, and G6 but not in G5. SIRU group G1 included four of the seven CC1 strains.

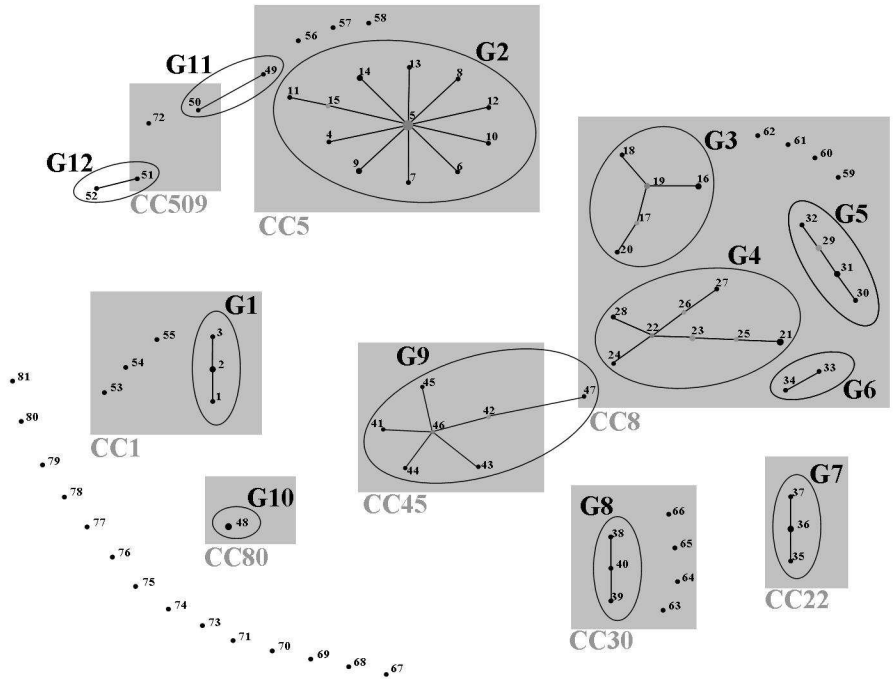

Figure 1. Schematic representation of the SIRU groups identified by eBURST v3 analysis. The size of the dots is proportional to the number of isolates of each SIRU profile. Single-locus variants are linked by lines; CCs are highlighted in gray.

Unexpectedly, two isolates which were totally different by all other typing methods were grouped in the same SIRU group, G12. In the same way, one CC8 strain appeared to be a single-locus variant of a CC45 strain and was therefore included in the same G9 SIRU group, and a single CC5 strain was clustered with a CC509 strain in G11.

Three of the 12 SIRU groups included a single PFGE type, as follows: G7, PFGE J; G8, PFGE K; and G10, PFGE L. Eleven of the 13 singletons defined by PFGE were also defined as singletons by SIRU typing.

Concerning *spa* typing, all SIRU groups contained related *spa* types, except for (i) one G4 strain (TAW166, t036), (ii) one G9 strain (HU109, t984), (iii) two G11 strains (CHL5, t535; and JP82, t375), and (iv) the two G12 strains.

Moreover, each *spa* type was associated with a single SIRU group, with the exception of t037, found in SIRU groups G4 and G6 belonging to the same CC, and t375, found in a G11 isolate and in singleton S20 (Table 1). Among the 10 different SCC*mec* types that characterized the MRSA collection (*n* = 78), with a few exceptions (Table 1), each SIRU profile was associated with a single SCC*mec* type.

Comparison of the discriminatory power of SIRU typing with that of other typing methods

The SID values obtained for the different typing methods are presented in Table 2. Considering the 104 isolates, the SIRU method showed a very high discriminatory power at the profile level (SID = 99.23%), similar to that observed for PFGE at the subtype level (SID = 99.85%) and higher than that observed for *spa* typing, considering both the length and sequence variation (SID = 97.61%). Considering discrimination of the SIRU method at the group level (SID = 93.24%), it was higher than that of MLST (SID = 91.77%) but lower than that of PFGE at the type level (SID = 95.41%). However, since the confidence intervals of the methods overlap (Table 2), we cannot exclude the hypothesis that they have similar discriminatory powers at a 95% confidence level.

TABLE 2. Number of types identified and Simpson Index of Diversity (SID) of each typing method for the 104 *S. aureus* isolates.

Typing method	No. of types identified	SID	95% CI[a]
PFGE subtype	97	99.85	(99.69-100.00)
SIRU profile	81	99.23	(98.66-99.81)
spa type	61	97.61	(96.36-98.86)
PFGE type	33	95.41	(94.11-96.70)
SIRU group	41	93.24	(90.62-95.87)
MLST - ST[b]	42	91.77	(88.52-95.01)
MLST - CC[c]	25	84.37	(79.68-89.06)

[a] CI, confidence interval
[b] multilocus sequence type - sequence type
[c] multilocus sequence type - clonal complex

Clustering concordance and directional agreement between SIRU typing and other typing methods

The clustering concordance between SIRU typing and the remaining methods (PFGE, *spa* typing, MLST, and SCC*mec* typing) could be traced based on the calculation of AR coefficients for the whole collection (Table 3). The AR values obtained for the SIRU typing method indicated that the highest level of congruence was at the group level for the ST ($AR_{SIRU\ group\text{-}ST}$ = 0.5736), followed by the CC ($AR_{SIRU\ group\text{-}CC}$ = 0.4963). At the profile level, the highest congruence was with *spa* type ($AR_{SIRU\ profile\text{-}spa\ type}$ = 0.4313). Congruence between PFGE (type and subtype levels) and SIRU typing was shown to be particularly low ($AR_{SIRU\ profile\text{-}PFGE\ type}$ = 0.1067; $AR_{SIRU\ profile\text{-}PFGE\ subtype}$ = -0.0025).

TABLE 3. Adjusted Rand and Wallace values for the entire collection (*n* = 104).

Typing method	Adjusted Rand		Wallace						
	SIRU group	SIRU profile	SIRU group	SIRU profile	PFGE type	PFGE subtype	*spa* type	MLST - ST[a]	MLST - CC[b]
SIRU group		0.1914		0.1113	0.2983	0.0138	0.2928	0.6713	0.9006
SIRU profile	0.1914		1.0000		0.4146	0.0000	0.9024	0.8293	1.0000
PFGE type	0.3180	0.1067	0.4390	0.0691		0.0325	0.1870	0.4959	0.7724
PFGE subtype	0.0242	-0.0025	0.6250	0.0000	1.0000		0.3750	1.0000	1.0000
spa type	0.4119	0.4313	0.8281	0.2891	0.3594	0.0234		0.8047	0.9453
MLST - ST[a]	0.5736	0.1289	0.5510	0.0771	0.2766	0.0181	0.2336		1.0000
MLST - CC[b]	0.4963	0.0800	0.3895	0.0490	0.2270	0.0096	0.1446	0.5269	

[a] multilocus sequence type - sequence type
[b] multilocus sequence type - clonal complex

The W coefficient was calculated to determine the capacity of SIRU typing to predict the classification achieved by other methods (Table 3). The results obtained showed that in this collection, two strains with the same SIRU profile have a 100% probability of belonging to the same CC, a 90% probability of sharing the same *spa* type, and an 83% probability of being classified in the same ST. The capacity of SIRU typing at the profile level to predict the PFGE type was low ($W_{SIRU\ profile\text{-}PFGE\ type}$ = 0.4146), and there was no correlation with the PFGE subtype ($W_{SIRU\ profile\text{-}PFGE\ subtype}$ = 0). On the other hand, the SIRU group showed a high correlation with the CC ($W_{SIRU\ group\text{-}CC}$ = 0.9006).

DISCUSSION

In the present study, we evaluated the performance of the SIRU typing method applied to a diverse collection of *S. aureus* isolates previously characterized by well-established typing methods. The SIRU method's typeability, i.e., the method's ability to assign a type to all isolates tested, was 89.4% when new primers designed for SIRU05 and SIRU16 locus amplification were used. The consistent nonamplification of SIRU05 from all strains belonging to ST1, CC22, CC45, and CC80 observed with our collection when we used the previously published SIRU05 primers is in agreement not only with previous studies focusing on SIRU typing of EMRSA-15 (CC22) isolates (29, 35-37) but also with the work of Ikawaty et al., who reported a reduced typeability for this specific locus on CC1, CC5, CC8, CC97, and CC228 isolates as well (39).

The ability of SIRU typing to assign a different type to two unrelated strains randomly sampled from the collection, i.e., its discriminatory power, was found to be very high (>99%) and was due mainly to the individual high levels of variability of SIRU05 and SIRU21 loci. High variability in SIRU21 is not surprising, since it is located in the known highly variable polymorphic region of the *spa* gene. The discriminatory power of SIRU typing at the profile level (SID = 99.23%) is similar to that of PFGE subtyping (SID = 99.85%) and *spa* typing (SID = 97.61%), considering the overlapping of the confidence intervals at 95%. Our results are concordant with a recent study, besides the fact that only six of the seven SIRU loci were taken into account (39). The observed high discriminatory power makes SIRU typing suitable for outbreak investigations, as also shown in studies by Hardy et al. where strains belonging to an outbreak or consecutively isolated from the same ward had the same or highly related SIRU profiles (35, 37). Moreover, in a study involving seven different outbreaks, variations in the number of SIRU repeats in strains belonging to the same outbreak were found to be rare (39).

The concordance between SIRU typing and well-established typing methods was measured by the calculation of the AR coefficient. We demonstrated that at the group level, SIRU typing showed the highest congruence with MLST (ST and CC), whereas at the profile level it showed the highest congruence with

spa typing. The correspondence with PFGE was low, in contrast to the study of Ikawaty et al. on 50 *S. aureus* isolates, where the AR coefficient between the MLVA method and PFGE (AR = 0.599) was higher than that for *spa* typing (AR = 0.435) (39).

The major CCs defined by MLST (CC5, CC8, CC22, CC30, and CC45) were maintained when the eBURST v3 algorithm was applied to our SIRU profile data. The exception was CC8, known to include a high degree of variability in STs, which was divided into four well-distinguished groups. Moreover, a single CC8 strain was surprisingly clustered with CC45 isolates (G9). Considering that MLST is based on the variation in housekeeping genes that have a slow evolutionary clock and that the SIRU method looks into variable repeat regions that could evolve more rapidly by an introduction or deletion of a single repeat, clonal types might be affected by genomic rearrangements to different extents in the two methods. The SIRU loci in noncoding regions are less likely to be subject to natural selection that affects some VNTRs located on coding regions or promoters (43). Interestingly, among the seven SIRU loci, different evolutionary clocks could be observed, since SIRU01, SIRU07, SIRU13, SIRU15, and SIRU16 appeared to be generally monomorphic between strains from the same group and therefore more conserved during evolution. SIRU05 and SIRU21 add high levels of variability to the method and could be especially informative for recent levels of evolutionary divergence. Therefore, the SIRU typing method includes different scales of evolutionary divergence within the same system, making it suitable for studies with different purposes.

The predictive power between SIRU typing and other methods, translated by the W index, was maximum for CC and very high for *spa* typing and MLST in our collection. In opposition, the prediction of the PFGE results was low, as SIRU typing was able to distinguish among isolates with the same PFGE subtype and vice versa. Noller et al. (49) showed that during an investigation of an *Escherichia coli* O157:H7 outbreak, MLVA appeared to have a sensitivity equal to that of PFGE and a specificity that was even superior to that of PFGE. On another hand, SIRU typing showed the highest congruence with MLST results, i.e., ST and CC results. It is noteworthy that the predictive power of a method seems to be linked

to the variability in the collection studied, as in a recent study involving two collections of *S. aureus* isolates, where for the first collection the MLVA method predicted the *spa* typing results but the reverse was not observed, while for the second collection both methods proved to be mutually predictive (39).

Our results show that SIRU typing analysis adds to the knowledge of the variability of the *S. aureus* genome and contributes to the understanding of genetic relationships among MRSA clones, as seen by Malachowa et al. (44).

In terms of convenience, the SIRU method was shown to be easy to perform, since it is based on single-locus PCRs, which could minimize the drawback of band size determination and even reduce the total time required for practical procedures if allied to the use of automated systems. Automated MLVA approaches were already proposed for *S. aureus* and *Staphylococcus epidermidis* genotyping through different PCR schemes (27, 28). In terms of cost, SIRU typing (11.2€/strain) is comparable to PFGE (13.2€/strain), but it is faster and technically easier to perform and much cheaper than *spa* typing (15.5€/strain) or MLST (80€/strain). Additionally, SIRU typing could be combined with *spa* typing, since the *spa* type may be determined by sequencing of the SIRU21 amplicons of nontypeable strains with conventional primers (1, 56).

In summary, as a PCR-based method, the SIRU typing method is relatively fast, accessible, and not expensive, which combined with its high discriminatory power makes it useful and reliable for short-term epidemiological investigations of *S. aureus*. In addition, its congruence with MLST results (at the CC and ST levels) makes it potentially valuable for evolutionary studies. In order for SIRU typing to be considered a useful tool in terms of epidemiological surveillance networks and evolutionary purposes, a public database similar to the databases available for other bacterial MLVA schemes (34) may be created for SIRU typing, allowing harmonization of *S. aureus* MLVA schemes and the interlaboratory exchange of data.

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Chapter VIII

General Discussion

Since the introduction of methicillin into clinical practice MRSA has emerged as a major human pathogen, initially exclusively in hospitals, and more recently in the community as well. However, MSSA, left for a second place facing the burden of MRSA, is now recognized not only as a colonizer, but as a virulent infectious agent as well. The present work described the genetic background of MSSA and MRSA populations from colonization and infection and in both hospital and community settings, giving new insights into: (i) the coexistence of MSSA and MRSA populations with different genetic backgrounds; (ii) the enhanced virulence and infectious potential of MSSA; and (iii) the clonal evolution and geographic spread of MRSA in national and international environments.

The validation of a new MLVA methodology constituted a major contribute of the present work on the continuous updating and validation of typing methods required for the characterization of *S. aureus* populations that are in permanent evolution.

MSSA: A COLONIZER BUT A CLINICAL THREAT

S. aureus carriage in different populations showed that the overwhelming majority of the isolates found in the anterior nares (ranging from 97% to 100%) are MSSA (7, 13, 17, 50, 72, 85) and several studies evidenced the endogenous source in many invasive infections. Moreover, successive reports of severe invasive disease due to MSSA, namely in children and neonates and otherwise healthy individuals are increasing (20, 34, 53, 58, 67, 68, 76);

Although the CDC is promoting a new national MRSA education initiative in the United States: ”Preventing MRSA skin infections” (http://www.cdc.gov/mrsa/mrsa_initiative/skin_infection/index.html#), 25% to 45% of the total community acquired *S. aureus* infections in two pediatric hospitals were due to MSSA associated to invasive infections (43, 54, 57).

MSSA as a virulent pathogen

MSSA infections are frequently associated to highly virulent strains producing several superantigens, hemolysins and leukocidins, namely PVL (73). Rasigade et al. reported a global distribution of PVL-positive MSSA pandemic lineages, responsible for a wide variety of diseases comprising SSTI, bone and joint infections, and necrotizing pneumonia (73). An unusually high (35%) prevalence of PVL-positive MSSA isolates was described in Cape Verde hospitals, in Chapter IV, compared to other studies on MSSA from nasal carriage and infection that reported a prevalence lower than 1.7% (44, 48, 52, 91).

In the Cape Verde MSSA collection, with the exception of four isolates recovered from skin wounds, all the remaining PVL-positive isolates were recovered from nasal colonization, which anticipates that infections would also be caused by these high virulent strains. Although the Portuguese MSSA population characterized in Chapter II showed some clonal overlapping with the MSSA population in Cape Verde, only two ST121 isolates from the former collection produced PVL, which suggests that hospitals in Cape Verde may represent a reservoir of PVL-positive clones. In addition, the acquisition of virulence determinants seems to confer a greater advantage to *S. aureus* than antibiotic resistance genes in a country and temporal period in which the antibiotic pressure was low due to the very limited use of antibiotics.

MSSA are also responsible for the majority of SSTI infections, namely cutaneous abscesses and cellulitis in Portuguese children attending a pediatric emergency unit, described in Chapter III. ST121 was the prevalent clonal type, but the strains were PVL negative in opposition to the Cape Verde isolates recovered 10 years earlier.

The ST121 MSSA lineage has been isolated in geographic distinct locations associated to both nasal carriage and disease, namely in children (10, 73, 92), and was already present among the early MSSA isolates from Denmark (32, 56), which demonstrates that it is a dynamic and evolving lineage. Of major concern was the recent report of methicillin resistance in ST121 isolates causing invasive disease in pediatric communities (16, 28).

Retrospectively, all genetic backgrounds found in Chapter III, among the Portuguese CA-MSSA causing disease (with the exception of ST152), had been previously described in carriage in the community between 1992 and 2001 (Chapter II), suggesting that although a high clonal diversity, MSSA lineages are more stable over time than MRSA (2). Moreover, the major CA-MSSA clones described in Europe (35) were also found in the pediatric Portuguese community (Chapter III) showing clonal spread similar to MRSA (53, 73).

A regular surveillance of SSTI, namely in children, is critical to predict and control the emergence of methicillin resistance and spread of staphylococcal infections in the community.

MSSA as a precursor of MRSA

The origin and evolution of MRSA is still a subject of study. Previous studies on the origins of MRSA support the multi-clone theory proposed by Musser and Kapur (62), suggesting a polyclonal nature of MRSA and the introduction of SCC*mec* several times into diverse MSSA lineages (25, 30, 32). Robinson and Enright (74) and Gomes et al. (32) proposed evolutionary models for the emergence of the five major MRSA clonal lineages currently spread worldwide: CC5, 8, 22, 30 and 45, based on multiple acquisitions of the SCC*mec* element by various MSSA backgrounds and in various occasions. Oliveira et al. designed an evolutionary pathway for the contemporary pandemic MRSA based on multiple and independent acquisition of the SCC*mec* by four distinct genetic backgrounds (65).

Molecular studies of the earliest MSSA isolates available from the Danish surveillance system from 1957 to 1972 showed that the genetic backgrounds of each of the actual major MRSA clones were already present in this Danish disease-causing isolates. However, the *mecA* gene was firstly aquired by only one of these genetic backgrounds, the Archaic clone (ST250) followed by a clonal expansion in hospitals (23, 32), rather than the multiple SCC*mec* acquisition.

In Chapters II and IV we characterized the population structure of MSSA from Portugal and Cape Verde, isolated from clinical and carriage sources. While

the Cape Verde collection included nosocomial isolates only, the Portuguese collection included isolates from both hospital and community. The two studies confirmed the high genetic diversity among MSSA populations (14, 24, 35, 36). However, despite the variability, 67% of the Portuguese MSSA and 46% of the Cape Verde isolates fell into four major clonal complexes (CC30, CC5, CC45 and CC8), overlapping with the major international pandemic MRSA lineages (25, 35).

In Cape Verde islands, no MRSA were found coexisting with the MSSA population. In Portugal, the major MSSA clones ST30, ST34, ST5 and ST45 showed some similarities with MRSA pandemic clones, i.e. EMRSA-16 (ST36), the New York/ Japan and Pediatric clones (ST5) and the Berlin clone (ST45) (Figure 1). However, none of these clones, with the exception of the Pediatric clone had been reported among nosocomial Portuguese MRSA isolates recovered during the same period, i.e. between 1992 and 2001 (3, 78). On the other hand, the genetic backgrounds corresponding to the three dominant MRSA clones in Portuguese hospitals until 2001 (Iberian-ST247, Brazilian-ST239, and EMRSA-15-ST22), or their ancestral genotypes (ST8 [ancestral of ST239 and ST247] and ST22), were not detected or only scarcely found among the co-existing MSSA collection. These observations resemble the early Danish scenario, where MSSA isolates belonged to genetic backgrounds that were later identified in different countries as major MRSA lineages, yet in Denmark only one of these backgrounds was represented at the time as the Archaic MRSA clone (19, 23, 32).

The Pediatric clone is the single MRSA clonal lineage that could have originated in Portugal through the acquisition of SCC*mec* type VI by the MSSA genetic background ST5. Therefore, our results suggest that the Iberian, Brazilian, and EMRSA-15 MRSA clones have not originated from the introduction of SCC*mec* into dominant MSSA backgrounds present in the Portuguese nosocomial or community environment but were more probably imported from abroad (Figure 1).

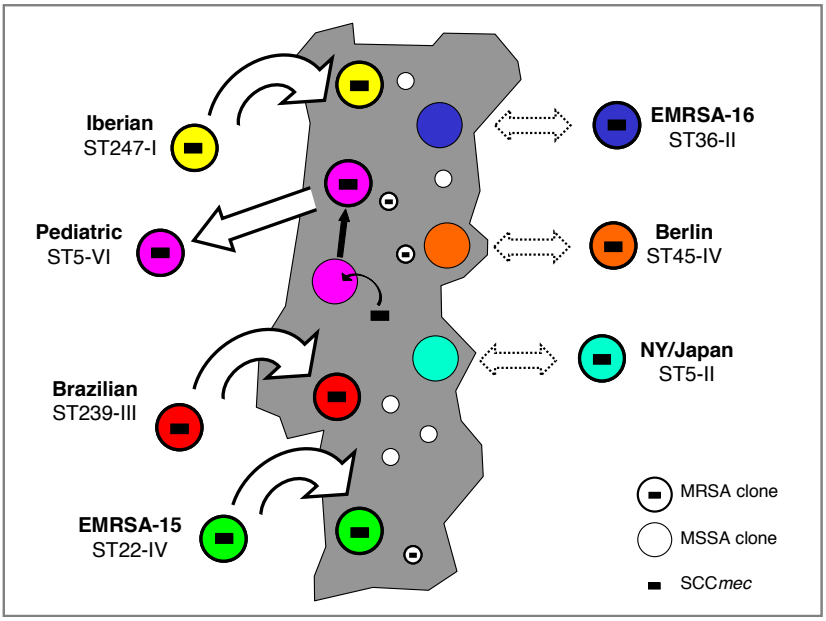

Figure 1. Schematic representation of the origin of the major MRSA clones in Portugal and similarities of the MSSA genetic background with foreign MRSA clonal lineages. Courtesy of M. Aires de Sousa, adapted.

The non-coexistence of MRSA in Cape Verde islands and the non-congruence between the genetic backgrounds of the major MSSA and MRSA clones within a population indicates that the presence of a successful MSSA lineage is not the sole factor necessary for SCC*mec* acquisition or for the success of an MRSA clone, which also supports the hypothesis that some genetic backgrounds may be more "receptive" of SCC*mec* than others (46). A "barrier" phenomenon was proposed to prevent the maintenance of the *mecA* in certain MSSA backgrounds limiting its evolution to a successful MRSA lineage (46). In fact, some genetic lineages with high virulent and epidemic potential such as ST121, associated to PVL, were already present in the 1960s and become a prevalent lineage maintaining the MSSA genetic background (32, 46, 73). Therefore, the introduction of SCC*mec* into sensitive clones seems to be a relatively infrequent and localized event compared to the geographical spread of MRSA clones. A recent study on HA-MRSA surveillance revealed that the New York/ Japan clone (ST5-II) emerged in Portuguese hospitals in 2006 (2). In Chapter V we described an MRSA isolate belonging to the Berlin clone (ST45) in the Portuguese Atlantic Azores islands as well as the Pediatric clone (ST5) as the

second most prevalent clone in the hospital in 2007-2008. These observations suggest that the introduction of SCC*mec* elements into local MSSA lineages could not be excluded.

The report of the ST5 background with different SCC*mec* cassettes (IVc and VI) in the Azores isolates suggests different acquisitions of SCC*mec* in the same MSSA ST5 background, which is in agreement with Nübel et al. description of the CC5 lineage evolution of temporal and geographic independent SCC*mec* acquisitions (63).

MRSA: STILL THE MOST FREQUENT RESISTANT PATHOGEN IN THE NOSOCOMIAL SETTING

The "Public consultation on strategies for improving patients' safety by prevention and control of health-care associated infections", by the European Commission in 2006 stated that "*Health-care associated infections affect an estimated one in ten patients and lead to considerable increase of illness, mortality and costs. These infections are not constrained by national boundaries and can rapidly spread between countries as evidenced by international spread of MRSA*" (27). In 2008, the European Antimicrobial Resistance Surveillance System reported for the first time a decreasing trend in the MRSA proportion in several European countries, and a global stabilization in MRSA prevalence. However, in contrast to the overall improvement, MRSA remains a serious problem in the Mediterranean region with Malta and Portugal showing MRSA proportions over 50% (26).

In the United States, the CDC reported that in 2006-2007, MRSA was responsible for 8% of all hospital acquired infections (42), while in Japan, 45.5% of all *S. aureus* recovered during 2004-2008 within a single hospital were MRSA (49), which requires a continuous nosocomial surveillance.

MRSA: filling the gap of Portuguese islands

With the exception of Malta, Portugal shows the higher invasive MRSA prevalence in Europe, which reaches 53% in 2008, and continues facing an increasing trend (26). Surveillance studies have been conducted in Portugal since the early 1990s, defining the clonal nature of the MRSA population over time. Successive waves of five major MRSA clones were described in Portuguese hospitals for a period of 16 years (2). The Portuguese clone (ST239-III variant) found in over 80% of the isolates in 1990-91 was almost completely replaced by the Iberian clone (ST247-I) during 1992-93. In 1999-2000, the Brazilian clone (ST239-III) was the prevalent clonal type being replaced by EMRSA-15 (ST22-IV) in 2003-2005. In 2006 emerged the first isolates belonging to the New York/ Japan clone (ST5-II) (2, 3, 9). These previous studies covered all the continental Portuguese territory, but the picture in the insular region was unknown. The MRSA collection described in Chapter V, recovered between 2007 and 2008 in the largest hospital of the Portuguese Azores archipelago, intends to fill this gap.

The clear dominance of the non-multiresistent EMRSA-15 clone (ST22-IV) in the Azorean hospital is, in part, in agreement with the situation in continental Portugal (2, 9). EMRSA-15 is also dominant in other European countries, namely the United Kingdom where it has been endemic for several years, but also in Asia and Australia (2, 9, 33, 45, 59). In fact, in a study involving isolates recovered in the same period from 11 hospitals scattered over continental Portugal, EMRSA-15 was found to be the most prevalent clone. However, and in contrast with the Azorean picture, the New York/ Japan clone (ST5-II) was shown to represent a new wave of MRSA in the country becoming the prevalent clone (2). Interestingly, in the neighbor Spanish islands of Majorca and Canarias EMRSA-15 was also found as a major clone, but not in the continental territory as in our country (8, 69, 90).

The clonal dissemination of *S. aureus* is mainly due to travel of human populations, as evidenced by the intercontinental spread of MRSA clones (4, 6). Hence, the frequent touristic flux from the United Kingdom, hypothesized as the cause of the importation of EMRSA-15 to the Mediterranean island of Malta (81), could also support, in part, the emergence of this clone in the most touristic

Azorean island. In parallel, the regular exchange of inhabitants between the islands and the continent seem to be an important vehicle of clonal importation.

The Pediatric clone (ST5-VI) was described for the first time in a Portuguese pediatric hospital in 1992, where it was dominant, but in the last national survey it was exceedingly rare (2, 78). Therefore, the hypothesis of a recent introduction of this clone in Azores from continental Portugal seems quite remote. On the other hand, the Azores islands have a high emigrant community in the United States, who frequently travel to the national territory, constituting also possible vehicles of dissemination of the Pediatric clone, frequently reported in the American continent (5, 31, 51, 78). Emergence of this clone locally is also plausible, since SCC*mec* types VI and IVc were found in the ST5 genetic background. There is evidence that ST5-MRSA emerged in numerous circumstances and in distinct locations, through multiple and independent acquisitions of SCC*mec* within ST5-MSSA lineage (63).

Interesting, the isolation of a single MRSA ST45 isolate carrying SCC*mec* IVa suggests another *de novo* SCC*mec* acquisition, probably in an already established MSSA ST45 background.

The exclusively detection of small SCC*mec* types (IV, V and VI) in the MRSA Azorean population reflects the actual tendency for the dominance of low resistant and fitness cost clones within the hospital setting, which parallels the community environment (15). Although the introduction of well-established community isolates into the hospital setting is not new, as reported for ST59 clonal type in Taiwan, no conclusions can be drawn for the flow direction in the Azores environment.

Additional insights into the MSSA and CA-MRSA populations co-existing in the geographic restricted Azores islands will be fundamental to support the proposed *S. aureus* localized evolution, as well as to understand the actual links between the hospital and the community.

MRSA population dynamics: clonal shifts over time

The massive geographic spread of MRSA results from the dissemination of a few highly epidemic clones (25, 35). Whereas cross-sectional studies showed

the predominance of one or two clones in a defined setting during the 1990's, several longitudinal studies showed a replacement of the predominant clones by others within a short temporal space, evidencing that the molecular epidemiology of HA-MRSA is dynamic (2, 12, 69, 94, 95).

Chapter VI describes the MRSA clonal evolution in Hungary, where the Hungarian MRSA clonal lineage (ST239-III) was massively spread in the nosocomial setting between 1993 and 1998 (22, 64). Soon after, the Hungarian clone decreased, and was progressively replaced by two other clones, both belonging to CC5, the Southern German (ST228-I) and the New York/ Japan (ST5-II), which became the prevalent clones in Hungarian hospitals.

The Southern German clone, first described in Germany in 1992, was soon detected in Budapest and then spread throughout the country. It was later frequently detected in other European countries, representing 86% of the MRSA isolates in Slovenia in 2004, and showing a similar increasing trend from 6% in 2002 to 28% in 2004 of the total MRSA in Finland (47, 61).

The New York/ Japan clone was first detected in the early 1990s as the dominant clone in Japan and soon after was reported as prevalent in the New York metropolitan hospitals evidencing a transcontinental spread. The highly epidemic potential of this ST5 clonal lineage was confirmed by the continuous reports of its prevalence in several hospitals in the United States and Canada, South America, eastern Asia and Australia (4, 18, 75, 83). The New York/ Japan clone had never been described in other European countries at the time of its emergence in Hungary, in 2001. Therefore, it was probably imported from another continent, suggesting a second transcontinental spread of this lineage.

We described for the first time the co-dominance and extensive spread of the New York/ Japan clone in a European country. Recent studies have reported the emergence of this clone in other European countries, such as Spain (70) and Portugal, where it started a new epidemic wave of MRSA in 2006 (2). Interestingly, in Portuguese hospitals, the New York/ Japan clone emergence was preceded by the massive spread of EMRSA-15 clone, which was only found as a minor clone in Hungary.

Clonal shifts were also observed in small geographic regions within a country, for instance in a small region of Switzerland, where within eight years, four distinct MRSA clones showed different relative proportions over time (12). Several studies from China, Mexico, Spain and Portugal described clonal evolution within single hospitals (9, 69, 89).

Local evolution of variants of well established clonal lineages as CC5 and CC8 were already reported, namely a new evolutionary MRSA lineage in Gregorian hospitals which resulted from an advantageous variable of the ST239 Brazilian clone (11). Additional evidence for a local evolution was the referred emergence of the Pediatric clone described in Chapter V, by the *de novo* introduction of a different type of SCC*mec* (type IVc) and the acquisition of PVL. Noteworthy, this is the first report of PVL among isolates belonging to the SCC*mec* type VI Pediatric clone.

In a recent report from a Portuguese hospital, changes in the antimicrobial policy were found to be related to the clonal nature of the MRSA population (9). However, neither the fundamental driving force promoting these clonal replacements nor the factors that lead to the high epidemic potential of specific clones is well understood.

MRSA in the Portuguese community: still not a problem?

Since the early 1990's that the emergence of specific MRSA clonal lineages causing infection in the community, in children and young otherwise healthy individuals without risk factors for hospital associated MRSA infection, makes a worrisome change in the MRSA epidemiology. CA-MRSA accounts for the overwhelming proportion of the MRSA infections in the United States and are responsible for the increasing trends in MRSA prevalence in The Netherlands and Northern European countries, were the HA-MRSA prevalence is contained by strict "search and destroy" policies. Major CA-MRSA PVL-positive pandemic lineages such as USA300 (ST8-IV), USA400 (ST1-IV), USA1000 (ST59-IV), the European clone (ST80-IV) and the Southwest Pacific clone (ST30-IV), are currently spread worldwide (86).

CA-MRSA is considered a serious and common public-health problem, translated by the increasing prevalence of SSTI due to MRSA. A report from the SENTRY Antimicrobial Surveillance Program in 1998-2004, identified *S. aureus* as a first cause of SSTI in North and Latin America and in Europe (55). In the United States the prevalence of SSTI infections increased dramatically since 1997 and a single CA-MRSA lineage, the USA 300, was found to be the predominant cause of SSTI in patients presenting at 11 emergency departments (41, 60). Similar increasing trends of CA-MRSA have been reported worldwide (21, 66).

Previous studies on the CA-MRSA prevalence in Portugal including isolates from nasal swabs of young healthy individuals and nasopharyngeal swabs of children attending day care centres, reported a very low MRSA prevalence in the Portuguese healthy community (0.24% in 1996-1998 and 0.13% in 2006-2009), suggesting that there is no CA-MRSA reservoir in these populations (77, 85). Since the nasopharynx is not the preferential niche of *S. aureus*, the true prevalence of MRSA was probably underestimated. Therefore, an alternative screening was carried out in Chapter III, including isolates from SSTI and swabs of the anterior nares of children attending a pediatric urgency unit in a Portuguese hospital. Although the prevalence of MRSA was still low in this population (3 MRSA out of 38 *S. aureus*), we reported the first European CA-MRSA isolate (ST80-IV PVL positive) in Portugal, which in contrast to what was described in several other European countries is not widely spread in this country (2, 29, 88). It is conceivable that the prevalence of CA-MRSA in Portugal is underestimated partially because skin infection samples are not routinely cultured.

TYPING METHODS: A CONSTANT NEED OF IMPROVEMENT

Typing is one of the most important tools to understand and control *S. aureus* infections. A panoply of typing strategies has been used in *S. aureus* studies, however no single typing method has proven to be exclusively sufficient to characterize a collection of isolates (84, 93). The choice of the most appropriate typing strategy largely depends on the study design and purpose, namely short-

term or long term surveillance, local or global epidemiology, routine surveillance or outbreak investigations. The currently most used typing methods for *S. aureus* characterization include PFGE, *spa* typing, MLST and SCC*mec* typing (for MRSA). Although regular updates have been introduced in these typing strategies, some drawbacks concerning ease of performance, portability of the results and associated costs, led to the improvement of new typing schemes such as MLVA (40, 71, 79, 80).

The staphylococcal interspersed repeat unit (SIRU) was the first MLVA scheme for *S. aureus* producing a string of numbers that characterizes each strain, which made the results amenable for interlaboratory comparisons similar to MLST (38, 40). Some of the performance criteria required for a typing method (87) were evaluated in Chapter VII, by the application of the SIRU methodology to a diverse collection of *S. aureus* isolates previously characterized by the most used typing methods. The high discriminatory power, similar to PFGE, made the SIRU method suitable for outbreak investigations, as also shown in studies by Hardy et al. where strains belonging to an outbreak or consecutively isolated from the same ward had the same or highly related SIRU profiles (37, 39). Moreover, the predictive power between the SIRU typing and the other typing methods, translated by the Wallace index, was maximum for clonal complex groupings and very high for *spa* typing and MLST.

SIRU typing includes different scales of evolutionary divergence within the same system, making it suitable for studies with different purposes, since some loci appeared to be generally monomorphic between strains from the same group and therefore more conserved during evolution, other loci add high levels of variability to the method and could be especially informative for recent levels of evolutionary divergence. One of these loci is SIRU21, which high variability was not surprising since it is located in the known highly variable polymorphic region of the *spa* gene. Therefore, SIRU typing could be easily combined with *spa* typing, since the later may be determined by sequencing of the SIRU21 amplicons in previously nontypeable strains (1, 82).

As a PCR-based method, the SIRU typing is relatively fast, accessible, and not expensive, which combined with its high discriminatory power and congruence

with MLST results (at the CC and ST levels) makes it useful and reliable for both short-term epidemiological investigations and long-term evolutionary studies.

Two additional MLVA schemes were recently proposed to overcome persistent drawbacks in terms of typeability, accuracy in amplicon sizing and harmonization of results. While Pourcel et al. (71) designed a more fastidious scheme including the amplification of 14 loci resolved by agarose gels, Schouls et al. (80) proposed a scheme of eight loci based in two PCR multiplex assays followed by an automated fragment sizing in a DNA sequencer apparatus.

Both schemes included some SIRU loci, namely SIRU21. In agreement with our results on SIRU typing, Schouls et al. found a high degree of concordance between MLVA groups and MLST clonal complexes, and a straightforward clustering of MLVA profiles compared to that of *spa* types. *spa* typing focuses on a single locus in the genome, and a small change, even a single mutation yields a different type, which sometimes is difficult to interpret in an evolutionary context. The use of multiple genome loci such as MLST or MLVA provides a more robust approach given that the relationship between two isolates that differ in one of the several loci tested is more trustful. Therefore, it is proposed that MLVA could outperform the currently well-established *spa* typing method for outbreak investigations, especially in hospital laboratories.

However, there is no consensus about a harmonized *S. aureus* MLVA scheme, which is mandatory for the construction of an online database to easily promote the interlaboratory exchange of data and make the MLVA method a promising tool in terms of epidemiological surveillance networks and evolutionary purposes.

CONCLUDING REMARKS

S. aureus has been showing an amazing capacity of adaptation to diverse environments and selective pressures. The study of different populations concerning methicillin resistance, colonization or infection status, both in the nosocomial and in the community settings, led us to reach new insights on the actual *S. aureus* epidemiological scenario.

Evolutionary trends were drawn in Chapter II, evidencing a most probable foreign origin of the nosocomial MRSA in Portugal, rather than the emergence in the co-existing MSSA population. The recent report in the hospital of CC5 and CC45 lineages (2), previously referred on the MSSA background, raises the question about the introduction of the SCC*mec* cassette into these MSSA lineages.

Although not all MSSA backgrounds seem permissive for SCC*mec* introduction (46), a few major well-adapted lineages become pandemic. Moreover, a worrisome scenario was painted in Chapters III and IV, describing well-armed MSSA lineages, carrying a panoply of virulence factors including PVL. Moreover, the amazing capacity of MSSA to promote disease in the pediatric community is of major concern, anticipating the birth of a new branch in the *S. aureus* pathogenesis: virulence instead of resistance. The increasing virulence of MSSA lineages is in line with the European and global scenario, warranting a new surveillance target.

In Portugal, in contrast to most European countries and the Unites States, despite the extraordinary high HA-MRSA prevalence, CA-MRSA seems to be still uncommon, even when the focus of the study was SSTI in the pediatric community, as described in Chapter III. The emergence and spread of MRSA in the community, where the antibiotic pressure is almost null, raises the question: “What manages the emergence of resistance outside the healthcare environment?”

The prevalence of the non-multiresistant EMRSA-15 in an Azorean hospital, described in Chapter V, highlights the epidemic potential of the clone in

detriment of high resistance. However, what drives the major epidemic potential of a *S. aureus* clone remains unclear.

Another unanswered question, regards the mechanisms driving the MRSA clonal replacement. In Chapter VI the 10-year surveillance study of MRSA in Hungarian hospitals clearly illustrates this phenomenon, which is in line with a plethora of clonal shift reports from all over the world. An intriguing point in the clonal evolution in Portuguese hospitals is the replacement of the previously prevalent EMRSA-15 by the New York/ Japan clone whereas in other European countries, namely the United Kingdom, this clone has been endemic in the hospitals in co-existence with the EMRSA-16, for a long time. The reasons underlying the distinct behavior of the same clonal lineage in different countries are not known and are certainly intriguing.

Although many questions are still unanswered and many other are emerging every day, the work developed and presented in this Thesis, filled some dark spaces on the knowledge of a spectacular “super bug” that constantly surprises us.

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