Update on the population structure of MRSA causing infection in a central hospital and in healthcare centers in Portugal

Diana Sofia Pereira Espadinha de Oliveira Costa

Dissertation to obtain a Master Degree in Medical Microbiology

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Abstract

Staphylococcus aureus is one of the most important human pathogens, being a major cause of infections worldwide both in the hospital and in the community.

In Portugal, the prevalence of methicillin resistant *S. aureus* (MRSA) in hospitals is one of the highest in Europe and has been characterized extensively; contrarily the prevalence and epidemiology of MRSA in the community has not been followed in a meaningful way.

To understand the epidemiological events that could explain a steep increase in MRSA frequency in a major Portuguese central hospital (HSM) within a 17 year period, two MRSA collections recovered in 1993 (n=54) and 2010 (n=180) from pus, blood and urine were analyzed by PFGE, MLST, *spa* and SCC*mec* typing. The results showed that a major clonal shift occurred, wherein ST22-IVh clone has replaced the previous ST239-IIIvar and ST247-I clones and accounts for more than 70% of the present population. Moreover, an increase in genetic diversity of MRSA clonal types was observed between the two study periods.

With the aim of determining the frequency and clonal nature of MRSA and methicillin-susceptible *S. aureus* (MSSA) causing skin and soft tissue infections (SSTI) in patients attending healthcare centers in Portugal, 73 samples were collected from nine healthcare centers (Medicos Sentinela Network). A total of 40 *S. aureus* were isolated, accounting for 55% of the SSTI, of which 17.5% were MRSA. MRSA isolates belonged to ST22-IVh (n=4), ST5-IVc (n=2) and ST105-II (n=1) that have also been described in the hospital in an equivalent period.

Our results suggest that the increase in MRSA frequency in HSM may be associated to the emergence of a MRSA clone with higher epidemic potential. Moreover, we propose that the spillover of MRSA from the hospital rather than community-associated-MRSA was the main cause of SSTI in persons attending healthcare centers in Portugal.

Resumo

Staphylococcus aureus é um dos principais agentes patogénicos humanos, sendo frequentemente associado a infecções nosocomiais e infecções na comunidade.

A prevalência de *S. aureus* resistentes à meticilina (MRSA) em hospitais portugueses é uma das mais elevadas da Europa e tem sido caracterizada extensivamente; contrariamente, a prevalência e epidemiologia de MRSA na comunidade em Portugal não tem sido devidamente seguida.

Com o objectivo de compreender as causas possíveis do aumento na frequência de MRSA num dos maiores hospitais centrais portugueses (HSM) ao longo de 17 anos, isolados de MRSA recolhidos em 1993 (n=54) e 2010 (n=180) de pus, sangue e urina foram analisados por PFGE, MLST, tipagem do *spa* e tipagem de SCC*mec*. Os resultados mostraram que ocorreu uma mudança global nos tipos clonais predominantes, onde o clone ST22-IVh substituiu os clones, ST239-IIIvar e ST247-I, representando mais de 70% da população actual. Além disso, entre 1993 e 2010 verificou-se um aumento na diversidade genética dos tipos clonais de MRSA.

Para determinar a frequência e a natureza clonal de MRSA e *S. aureus* sensíveis à meticilina (MSSA) isolados de infecções de pele e tecidos moles (SSTI) em pessoas que frequentam centros de saúde em Portugal, 73 amostras foram recolhidas em nove centros de saúde (Rede Médicos Sentinela). Isolou-se um total de 40 *S. aureus* (55%), dos quais 17,5% eram MRSA. Os isolados de MRSA pertenciam aos clones ST22-IVh (n=4), ST5-IVc (n=2) e ST105-II (n=1), que foram descritos neste estudo como sendo clones de origem hospitalar.

Os nossos resultados sugerem que o aumento da frequência de MRSA no HSM pode estar associado à emergência de um clone de MRSA com maior capacidade epidémica. Além disso, verificámos que a principal causa de SSTI em pessoas que frequentam centros de saúde em Portugal são MRSA de origem hospitalar e não MRSA associados à comunidade.

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Abbreviations List

ACME – arginine catabolic mobile element

BURP – Based Upon Repeating Pattern

CA-MRSA – community-associated methicillin-resistant *Staphylococcus aureus*

CC – clonal complex

CO-MRSA – community-onset methicillin-resistant *Staphylococcus aureus*

CoNS – coagulase-negative staphylococci

DLV – double locus variant

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

eBURST – electronic Based Upon Related Sequence Types

HA-MRSA – hospital-associated methicillin-resistant *Staphylococcus aureus*

HSM – Hospital de Santa Maria

LA-MRSA – livestock-associated methicillin-resistant *Staphylococcus aureus*

MDR – multidrug-resistance

MIC – minimal inhibitory concentration

MLST – multilocus sequence typing

MRSA – methicillin-resistant *Staphylococcus aureus*

MS – Médicos Sentinela

MSSA – methicillin-susceptible *Staphylococcus aureus*

ORF – open reading frame

PBP – penicillin-binding protein

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PVL – Panton-Valentine Leukocidin

SCC*mec* – staphylococcal chromosome cassette *mec*

SLV – single locus variants

spa – *Staphylococcus aureus* protein A

ST – sequence type

TLV – triple locus variant

Chapter 1. Introduction

1.1 *Staphylococcus aureus* – Historical Perspective

Staphylococcus aureus is the most important pathogen in the *Staphylococcus* genus. It was first described in 1880, by Louis Pasteur and Alexander Ogston, as small spherical bacteria causing pus from furuncles and abscesses, having been called micrococci. However, Ogston was the first to demonstrate that these bacteria produced inflammation and suppuration and introduced the name *Staphylococcus* derived from the Greek *staphyle* (from bunch of grapes). In 1884, Rosenbach provided the first taxonomic description of the genus *Staphylococcus* based on the colonies' pigmentation, dividing the genus in two species, *Staphylococcus aureus* (*aureus* from the Latin word for gold) and *Staphylococcus albus* (*albus* from the Latin word for white), later renamed *Staphylococcus epidermidis* (94).

1.2 The *Staphylococcus* genus

The genus *Staphylococcus* comprises more than 40 species and several subspecies (http://www.bacterio.cict.fr/s/staphylococcus.html), all of them sharing some common characteristics. Morphologically they are gram-positive cocci of approximately 0.5-1.5 μm of diameter, dividing in three perpendicular planes to form grape-like clusters. These bacteria have a distinct cell wall composed of teichoic acids and a peptidoglycan characterized by several layers of N-acetylglucosamine and N-acetylmuramic acid linked by a β-(1,4)-glycosidic bond and cross-linked by five glycine residues in the interpeptide bridge, in the case of *S. aureus*, which renders them susceptible to lysostaphin (112).

Staphylococci are nonmotile, nonflagelate, facultative anaerobes, glucose fermenting, catalase-positive and usually oxidase-negative. They are also characterized by their tolerance to high concentrations of NaCl, with the majority being able to grow in the presence of 1.7 M of NaCl. Staphylococci are among the hardiest non-spore forming bacteria, resisting to drying and to heat (as high as 60ºC for 30 min). Staphylococci are also characterized by having a low G+C content (30-38%) (135, 200).

1.3 The *Staphylococcus aureus* species

Staphylococcus aureus is characterized by its capacity to produce the coagulase enzyme or clumping factor, which is a cell surface-associated fibrinogen-binding protein that, in the presence of human plasma, causes agglutination. This property is useful for distinguishing *S. aureus* from other staphylococci that are unable to produce coagulase and that are referred to as coagulase-negative staphylococci (CoNS) (135). Also, *S. aureus* is capable of fermenting mannitol aerobica- and anaerobically. When grown on mannitol salt agar, a yellow zone is formed around the colony due to the fermentation of mannitol and production of acid. Additionally, *S. aureus* can also metabolize glucose, xylose, lactose, sucrose, maltose and glycerol.

In complex media, *S. aureus* is able to grow within a wide range of pH (4.8-9.4) and temperature (10º to 45ºC), showing a minimal doubling time of 30 to 40 min (135).

1.4 *Staphylococcus aureus* colonization and pathogenicity

S. aureus is a commensal organism that is frequently found colonizing the skin and mucosas of mammals but can also be found in birds. In humans, its primary niche for colonization are the anterior nares although it can be found colonizing other parts of the body such as the pharynx, hands, forearms, gastrointestinal tract, perineum, vagina and axillae, but in a lower frequency (193, 198).

Overall, three patterns of nasal carriage can be distinguished: ~20% (range 12-30%) of people are persistent carriers, ~60% (range 16-70%) are transiently carriers and another 20% (range 16-69%) are non-carriers of *S. aureus* (95). The pattern of nasal carriage seems to depend on a complex interplay between bacterial factors and host characteristics (53, 187). Importantly, some studies show that persistent carriers are at a higher risk of developing infection than non-carriers (134, 149, 199). Transmission of *S. aureus* can be accomplished by direct contact (e.g., skin-to-skin contact with an infected or colonized person), by indirect contact (e.g., contact with a contaminated surface or object) or by airborne transmission (193).

Some groups within the general population seem to be at a higher risk for *S. aureus* colonization, presenting increased *S. aureus* carriage rates. In these groups are included:

hospitalized patients, healthcare workers, patients with diabetes mellitus (both insulin dependent and non-insulin dependent), patients undergoing haemodialysis or continuous peritoneal dialysis, HIV patients, patients with viral infections of the upper respiratory tract, patients with *S. aureus* skin infections and skin disease (e.g., eczema or psoriasis), obesity, history of cerebrovascular accidents and intravenous drug users. More recently, veterinary staff, pet owners and (pig) farmers have also been considered at risk for *S. aureus* colonization (110, 181, 190, 198).

Because *S. aureus* is capable of causing infection when the skin and mucous membranes become disrupted, it is also considered an opportunistic pathogen. Infection caused by *S. aureus* can range from mild conditions, such as skin and soft tissues infections (e.g., cellulitis, folicullitis, mastitis, impetigo, furuncles, superficial and deep skin abscesses, wound infections), to severe life-threatening diseases (e.g., pneumonia, meningitis, bacteremia, endocarditis, osteomyelitis) and toxin-mediated diseases (e.g., toxic shock syndrome, scalded skin syndrome and food poisoning) (112, 181).

1.5 Antibiotic resistance in *Staphylococcus aureus*

Prior to the antibiotic era, the mortality rate of *S. aureus* invasive infections was extremely high, exceeding 80% (171). However, in the early 1940s, with the introduction of the first ß-lactam antibiotic named penicillin, the patients' prognosis improved considerably. Unfortunately, shortly after the introduction of penicillin into medical practice, the first resistant isolates soon emerged. This resistance was associated to the production of a plasmid-encoded ß-lactamase enzyme (1). Since then, resistance to penicillin has been progressively increasing. Nowadays, more than 90% of staphylococcal isolates produce ß-lactamases (111).

Soon after the introduction of penicillin, other antimicrobial agents were developed and used to treat *S. aureus* infections, including chloramphenicol, erythromycin, streptomycin and tetracycline. Nevertheless, resistance to these antimicrobials rapidly emerged, often mediated by plasmids and transposons (107).

In the beginning of the 1960s new drugs emerged like the first cephalosporins, gentamicin and more importantly, the first semi-synthetic ß-lactamase resistant

penicillin, called methicillin (and later also oxacillin). However, within two years of the introduction of methicillin in clinical use, the first methicillin-resistant *S. aureus* (MRSA) were reported (86). Since then, multidrug-resistant MRSA became a major health problem in hospital, frequently accumulating and developing resistance to every class of antimicrobials and spreading worldwide (45). Currently, due to the multiple resistance often presented by healthcare-associated MRSA (HA-MRSA) in hospitals, therapeutic choices are limited. Glycopeptides, namely vancomycin, is one of the few antibiotics effective against these microorganisms, being considered a last resort antibiotic. Nevertheless, in 1996, the first vancomycin-intermediate *S. aureus* (VISA) strain was reported in Japan (80) followed in 2002, by the first vancomycin-resistant *S. aureus* strains reported in the USA (28). Importantly, in 2008 the first VISA strain from a Portuguese hospital was also reported (64).

While HA-MRSA became one of the major pathogens in the nosocomial setting, in the mid 1990s MRSA began to cause infections in the community in previously healthy people (27, 186). However, these MRSA strains, designated as community-associated MRSA (CA-MRSA) were distinct from HA-MRSA, having a different genetic background and being frequently susceptible to non-β-lactams classes of antimicrobials (41).

Alternative drugs have been developed to treat MRSA infections (108), however these are generally expensive (e.g., quinupristin-dalfopristin, tigecycline, daptomycin and linezolid) or reveal a high level of toxicity to the patients (e.g., rifampicin), and resistance to these antimicrobials has already been observed. Therefore, the development of new antimicrobial agents and, more importantly, new measures to prevent infections and to control the spread of MRSA are warranted.

1.5.1 ß-lactam antibiotics and mechanisms of resistance

The ß-lactams are a broad class of antibiotics, which the primary target are the PBPs (penicillin-binding proteins) that are involved in the final stages of peptidoglycan synthesis. The PBPs (1 to 4) are responsible for the transglycosylation and/or transpeptidation reactions. ß-lactam antibiotics bind to the native PBPs present in the wall of susceptible *S. aureus*, inactivating their transpeptidase domain. By this way, the

final stages of the cross-linking in the peptidoglycan are inhibited, consequently leading to bacterial death (17).

In *S. aureus*, resistance to ß-lactam antibiotics can occur mainly by two mechanisms. One mechanism, conferring resistance only to penicillin, is based on the production of the ß-lactamase enzyme encoded by the *bla*Z gene, which cleaves the beta-lactam ring of penicillin, rendering it ineffective (116). Another broader resistance mechanism conferring resistance to most ß-lactams is based on the acquisition of an exogenous resistance determinant, the *mecA* gene (78). Due to its clinical and epidemiological importance, this mechanism will be further described.

Other mechanisms of resistance to ß-lactams were also described, including the hyperproduction of ß-lactamase enzyme (116) and the alteration of the native PBPs by mutation (183).

1.5.2 Methicillin resistance: the *mecA* gene and the staphylococcal cassette chromosome *mec* (SCC*mec*) element

The central element conferring resistance to methicillin and all other ß-lactam antibiotics is the *mecA* gene (2.1kb in length), which encodes for a penicillin-binding protein, PBP2a or PBP2', with low affinity to all ß-lactam antibiotics (78). In the presence of ß-lactam antibiotics the transpeptidase domain of the four *S. aureus* native PBPs is inactivated but the transpeptidase domain of PBP2a remains active, which together with the transglycosilase of the native PBP2, ensures the synthesis of peptidoglycan (153).

The *mecA* gene is carried in a mobile genetic element named staphylococcal cassette chromosome *mec* (SCC*mec*) that integrates at the 3' end of *orfX*, an open-reading frame (ORF) with unknown function located in the *S. aureus* chromosome near the origin of replication (82, 89). SCC*mec* is composed by: (i) the *mec* gene complex containing *mecA* and its regulators; (ii) the *ccr* gene complex containing site-specific recombinases - cassette chromosome recombinases (*ccr*) and (iii) the presence of flanking direct and inverted repeats (85).

According to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), the SCC*mec* elements are defined by the combination of the class of the *mec* gene complex and the *ccr* gene complex.

So far, five major classes of *mec* gene complex were described in *S. aureus*: *mec* complex A, B, C (C1 and C2) and E (see Table 1) (85). An additional *mec* complex class, class D, was described in coagulase-negative staphylococci (CoNS) (88) but so far was not found in *S. aureus.*

Table 1 – Structure of *mec* gene complex classes currently described and associated SCC*mec* types. Adapted from (**http://www.sccmec.org)**

***mec* gene complex**	**Structure**	**SCC*mec* types**
class A	IS*431-mecA-mecR1-mecI*	II, III, VIII
class B	IS*431-mecA*-Δ*mecR1*-IS*1272*	I, IV, VI
class C1	IS*431-mecA*-Δ*mecR1*-IS*431* (two IS*431*s are arranged in the same direction)	VII, X
class C2	IS*431-mecA*-Δ*mecR1*-IS*431* (two IS*431*s are arranged in the opposite direction)	V, IX
class D	IS*431-mecA*-Δ*mecR1*	*
class E	*blaZ-mecA*$_{LGA251}$-*mecR1*$_{LGA251}$-*mecI*$_{LGA251}$	XI

* No SCC*mec* was attributed.

The *ccr* gene complex is composed by the *ccr* genes and surrounding open reading frames, several of which have unknown functions. The *ccr* genes encode for recombinases belonging to the invertase/resolvase family and are responsible for the excision/integration of SCC*mec* from/into the chromosome (85, 89). Currently, three phylogenetically distinct *ccr* genes (<50% of nucleotide identities) have been described: *ccrA*, *ccrB* and *ccrC*, where *ccrA* and *ccrB* can be further differentiated in allotypes (nucleotide identities between 60% and 82%).

Two structures of *ccr* complex can be identified: one carrying two adjacent genes, *ccrA* and *ccrB*, and the other carrying only one gene, *ccrC*. Eight *ccr* gene complex types were already described (see Table 2): types 1 to 4, carrying two genes, *ccrA* and *ccrB* with four allotypes: *ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrAB4*; type 5 carrying a single allotype,

ccrC1; type 7 carrying the combination of *ccrA1B*6 and type 8 carrying the combination *ccrA1B*3 (http://www.sccmec.org). Additionally, *ccr* type 6 (*ccrA5B6*) was found in a CoNS isolate strain but not in *S. aureus* (208).

Table 2 – Structure of *ccr* gene complex types and associated SCC*mec* types currently described. Adapted from (**http://www.sccmec.org**)

***ccr* gene complexes**	**Structure (allotypes)**	**SCC*mec* types**
Type 1	*A1B1*	I, IX
Type 2	*A2B2*	II, IV
Type 3	*A3B3*	III
Type 4	*A4B4*	VI, VIII
Type 5	*C1*	V, VII
Type 6	*A5B3*	*
Type 7	*A1B6*	X
Type 8	*A1B3*	XI

*No SCC*mec* was attributed.

Up until now, eleven SCC*mec* types were identified in *S. aureus*: SCC*mec* type I (class B *mec* complex, *ccrA1B1*), SCC*mec* type II (class A *mec* complex, *ccrA2B2*), SCC*mec* type III (class A *mec* complex, *ccrA3B3*), SCC*mec* type IV (class B *mec* complex, *ccrA2B2*), SCC*mec* type V (class C2 *mec* complex, *ccrC1*), SCC*mec* type VI (class B *mec* complex, *ccrA4B4*), SCC*mec* type VII (class C1 *mec* complex, *ccrC1*), SCC*mec* type VIII (class A *mec* complex, *ccrA4B4*), SCC*mec* type IX (class C2 *mec* complex, *ccrA1B1*), SCC*mec* type X (class C1 *mec* complex, *ccrA1B6*) and SCC*mec* type XI (class E *mec* complex, *ccrA1B3*) (18, 63, 81-83, 85, 102, 113, 142, 205).

In addition to *mec* and *ccr* gene complexes, the SCC*mec* element also contains three joining regions (J1, J2 and J3) (previously named "junkyard regions"), which are nonessential components of the cassette that may carry pseudogenes or additional antimicrobial resistance determinants encoded in mobile genetic elements such as insertion sequences, transposons and plasmids (85).

The J1 region is located between the right chromosomal junction and the *ccr* complex; the J2 region is located between the *ccr* gene complexes and the *mec* gene complex; and

the J3 region is between the *mec* complex and the left chromosomal junction. Overall, SCC*mec* structure can be summarized as containing a J1-*ccr*-J2-*mec*-J3 structure, where the variations and polymorphisms within the same combination of *mec* and *ccr* complexes are used to define SCC*mec* subtypes (85, 121).

In the last years SCC*mec* type IV has raised particular interest. SCC*mec* type IV is the smallest structural type of SCC*mec* and believed to be the most mobile version. It has been found among community-associated MRSA strains (CA-MRSA) (204) and is characteristic of some important nosocomial MRSA clones, such as the EMRSA-15 clone, which is endemic in the UK (87) and is spreading in many other countries (8, 9, 120), including Portugal (4, 11, 43). So far, ten SCC*mec* type IV subtypes (IVa through IVl) have been described, based in the variations of J1 region only (19, 84, 121).

Currently, the definition of MRSA clone is based on the SCC*mec* type carried by the isolate and its clonal background (sequence type - ST) defined by multilocus sequence typing (MLST), as proposed by Enright *el al.* (55).

1.6 Virulence factors and their role in pathogenesis in *S. aureus*

S. aureus has a wide variety of virulence factors that play an important role in the establishment of infection and pathogenesis.

These virulence factors may be involved in different stages of infection and can be grouped into: (i) attachment and adherence to host tissues (e.g., clumping factors, fibronectin-binding proteins); (ii) tissue invasion/penetration (proteases, lipases, nucleases, hyaluronate lyase and metalloproteases) (iii) evasion and destruction of host defenses [cytolysins (e.g. phenol-soluble modulins (PSMs), capsular polysaccharides, protein A, chemotaxis inhibitory protein (CHIPS)] and (iv) persistence in the host (e.g., biofilm accumulation) (69).

S. aureus is also able to produce a myriad of toxins such as: enterotoxins (SEA to SEQ) responsible for food poisoning, superantigenic toxins [e.g., toxic shock super toxin-1 (TSST-1)], responsible for toxic shock syndrome, and epidermolysins or exfoliative toxins (ETA, ETB, ETC and ETD) the major causes of bullous impetigo and staphylococcal scalded skin syndrome (SSSS) (69).

1.6.1 Panton Valentine Leukocidin – PVL

More recently, staphylococcal cytolysins, namely Panton-Valentine leukocidin (PVL) have been demonstrated as key determinants of *S. aureus* infections.

Panton Valentine Leukocidin (PVL) is a *S. aureus* two-component pore forming protein that belongs to the family of synergohymenotropic toxins. PVL was first described by Van deVelde in 1894, although Panton and Valentine were the first to associate it with skin and soft tissue infections (SSTI) in 1932 (41).

PVL is encoded by the *lukPV* operon, comprising the co-transcribed *lukF-PV* and *lukS-PV* genes, and carried by several temperate bacteriophages (21, 207). Being a leukocidin, PVL has the ability of forming pores in the membranes of human polymorphonuclear neutrophils (PMNs), monocytes, and macrophages, consequently leading to their lysis or apoptosis, in a concentration-depending manner (23, 147).

PVL has been strongly associated with CA-MRSA infections in several epidemiological studies, being considered one of the molecular markers for CA-MRSA. Although strong and unequivocal epidemiological correlation between the presence of PVL and the severity of disease exist, still the increased virulence potential of PVL-positive MRSA strains has been difficult to prove due to discordant results obtained for different animal and infection models (23, 49, 101, 104, 195). So far, PVL was already found associated with several different clones (STs) and genetic backgrounds (CC) such as CC5, CC8, CC15, C22, CC30, CC59, ST45, ST72, CC80, CC88, ST93, ST154, ST398 and ST772 (127).

1.6.2 Arginine catabolic mobile element – ACME

ACME is a 30.9 kb DNA chromosomal encoded element that has been hypothesized to play a role in the pathogenesis of MRSA, especially CA-MRSA, by enhancing its ability to colonize the skin of healthy people and, in that way, to disseminate in the community (50). ACME comprises two main gene clusters, the *arc* genes and *opp3* operon, and three allotypes have been described so far in staphylococci. ACME type I, which has been strongly associated with ST8-IV strains (USA300 clone) (47), contains the two gene clusters, *arc* and *opp3*, whereas ACME type II only contains the *arc* cluster and ACME type III only contains the *opp3* cluster. The ACME types I, II and

III, as well as some variants of these allotypes, were described in *S. epidermidis* (14, 125).

The *arcA* gene encodes an arginine deiminase that inhibits nitric oxide production, a metabolite produced by human macrophages and other leukocytes in response to bacterial infections. Also, it seems to play a role in ATP production and in survival at low pH (4.2-5.9), which is the pH usually found in the skin and in the intracellular milieu. The *opp3* cluster encodes a putative oligopeptide permease operon (*opp*) that belongs to the ABC transporter family, which can be associated to a wide array of functions, including peptide nutrient uptake, quorum sensing, pheromone transport, chemotaxis, eukaryotic cell adhesion, binding of serum components and expression of virulence determinants (47, 48, 147). However, the real contribution of this element to the virulence of CA-MRSA has still to be clearly demonstrated. A study by Diep *et al.* (50) showed that USA300 had a superior fitness compared to its isogenic mutant with ACME and SCC*mec* deleted, suggesting that ACME may be advantageous. In contrast, a study by Montgomery *et al*. (130) did not show any difference in virulence between USA300 strains and its isogenic ACME knock-out mutant in a rat model of necrotizing pneumonia or skin infections. Therefore, the role of ACME in USA300 pathogenesis remains controversial.

ACME integrates into *orfX* and is flanked by repeat sequences characteristic of SCC*mec* elements but does not contain *ccr*. Its mobilization is believed to be achieved by the recombinases encoded by SCC*mec*, namely the *ccrAB*2 complex present in SCC*mec* type IV that is normally found in tandem with ACME in *S. aureus* (50).

ACME has been found in different clonal backgrounds, other than ST8-IVa, such as ST5-II (50, 66), ST59-IVa (50), ST97-V (51), ST1-IVa (51), ST5-IV (52), ST239-III (65), also two ST8 MSSA isolates (66) and in some species of coagulase-negative staphylococci, where it seems to be more prevalent (14, 125, 152). More recently, a novel type of ACME was described in ST22-IV (169) that has significant homology to ACME II found in *Staphylococcus epidermidis*, which reinforces the hypothesis that ACME might have been acquired through horizontal transfer and recombination events occurring between CoNS and *S. aureus* (47, 169).

1.7 Typing of *Staphylococcus aureus*

S. aureus is one of the most important human pathogens in the hospital setting with an increasing importance in the community as well. Therefore, the efficient monitoring and control of this pathogen is crucial, which requires efficient typing tools. In order to better understand the epidemiology and the molecular evolution of this pathogen, several typing techniques have been developed throughout the years, each one providing a characterization of the pathogen at different levels in terms of evolutionary time clock and discrimination power, and targeting different loci, according to the purpose of the study.

1.7.1 PFGE

Pulsed-field gel electrophoresis (PFGE) is one of the most discriminatory typing methods for *S. aureus*, having been considered for a long time the gold standard method to investigate MRSA nosocomial outbreaks and also to describe the local and worldwide dissemination of *S. aureus* clones (37, 44).

PFGE was first described in 1984 to analyze chromosomal DNA of eukaryotic organisms (165) and afterwards has proven to be applicable to several other organisms, namely bacteria, including *S. aureus* (106).

PFGE involves the embedding of the bacterial cells in agarose, their lysis *in situ* and the digestion of the bacterial total DNA with a low frequency cutting restriction endonuclease, generating a small number of DNA fragments. These DNA fragments are resolved in a special electrophoretic apparatus, in which the electric field is applied in pulses that vary in time and orientation according to a predetermined pattern, producing a macro restriction pattern of distinct bands (12).

For the interpretation of the macro restriction patterns obtained by PFGE, consensus guidelines allow drawing conclusions on epidemiological relatedness based on the comparison of PFGE macro restriction profiles. According to these *S. aureus* established guidelines, for an outbreak situation an isolate is considered to be closely related to the outbreak strain if its restriction pattern differs from the outbreak pattern by changes consistent with a single genetic event, i.e., a point mutation or an insertion or

deletion of DNA, resulting in two to three band differences; possibly related to the outbreak strain if restriction pattern differs from the outbreak pattern by changes consistent with two independent genetic events (i.e., four to six band differences); and unrelated to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with three or more independent genetic events (generally seven or more band differences) (115, 180). However, nowadays the analysis of the macro restriction patterns is usually assisted by bioinformatics tools, such as Bionumerics software (v6.1 Applied Maths, Saint- Martens-Latem, Belgium) where a set of guidelines (e.g., image acquisition, internal controls, data range for analysis) are taken into account so that the method of analysis can be standardized and applicable intra and inter-laboratory (26, 58).

PFGE has been considered a highly discriminatory and reproducible technique with a high typeability. However is a time-consuming and laborious technique. Moreover, the interlaboratory comparison and standardization are difficult to achieve (176), although several attempts have been made in order to harmonize the procedures and improve data interchangeability (32, 37, 131, 189)

1.7.2 *spa* typing

Currently, a widely used technique is *spa* typing that was initially described by Frenay *et al.* (62). This technique is based on the sequencing of the polymorphic X region or short sequence repeat (SSR) of *S. aureus* protein A gene (*spa*), a surface protein anchored in the cell membrane of most *S. aureus* that has the ability to bind selectively to the fragment crystallizable (Fc) region of immunoglobulin G (IgG), leaving the antigen-combining sites free, in this way preventing opsonization (69). This polymorphic X region consists of a variable number of small repeats, usually of 24bp (although 21, 27 and 30bp repeats were already described) (http://spaserver.ridom.de) and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. Even though the X region presents a high diversity due to point mutations, deletions and duplications of the repetitive units, its flanking regions are well conserved allowing the annealing of primers and the direct amplification and sequencing of this region (167).

To the sequence of each different repeat an alphanumerical code is attributed and to the combination of these repeats a specific *spa* type is assigned (76). Two different nomenclature systems used for *spa* typing were developed (76, 97). The one more widely used uses the Ridom software for automatic analysis and type attribution (Ridom GmbH, Wurzburg, Germany); it has proven to be a valuable tool, for the comparison of *spa* typing data using a uniform nomenclature while providing an excellent quality control of the data (2). All *spa* data is synchronized to an online database, the SpaServer (http://spaserver2.ridom.de/index.shtml), for the assignment of the *spa* repeats and *spa* types. The European SeqNet network (http://www.seqnet.org) is responsible for the curation of the SpaServer database, being one of the largest databases for *S. aureus* typing.

Overall, *spa* typing has a high reproducibility, typeability and a good discriminatory power [between that of PFGE and multilocus sequence typing (see below)] (176) and one of its greatest advantages is the easy data exchangeability (2).

Because of its capacity to cover genetic variations that accumulate both rapidly and slowly changes (micro- and macro-variation), *spa* typing is considered a valuable tool for outbreaks, local and global epidemiology, and population-based studies (97). It has also the advantages of being less expensive, less laborious and time consuming and easy to perform, as compared to MLST and PFGE, since involves sequencing of a single locus (168, 174). Furthermore, the implementation of the algorithm based upon repeat pattern (BURP) (118), in association with StaphType, allowed the establishment of phylogenetic relatedness using *spa* typing data, enabling the clustering of *spa* types into clonal lineages with a good concordance with other typing methods such as MLST (CC) and PFGE types (37, 58, 75, 97, 174, 175).

1.7.3 Multilocus sequence typing - MLST

MLST was first described in 1998 (114) for the characterization of *Neisseria meningitidis* lineages and was afterwards adapted to other microorganisms including *S. aureus* (56). MLST is a molecular typing technique based on the nucleotide sequencing of an internal region of seven housekeeping genes. For *S. aureus,* the genes analyzed by MLST scheme are the following: carbamate kinase (*arcC*), shikimate dehydrogenase

(*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) (54).

For each gene sequence a distinct allele is assigned; the combination of the seven alleles defines an allelic profile to which a sequence type (ST) is attributed. The ST assignment (e.g., ST22, allelic profile: 7-6-1-5-8-8-6) is performed through an open access database on the Internet (www.mlst.net), in which the sequences obtained from isolates all over the world can be deposited and compared.

To analyze and compare MLST data an algorithm named BURST or eBURST (from electronic based upon related sequence types) was developed (59). This tool is freely available on the Internet (http://eburst.mlst.net) and allows the determination of the pattern of descendance among a particular population (55, 59).

The algorithm eBURST is based on the theory that a genotype within a population, whether by a fitness advantage or random genetic drift, eventually can become predominant and then gradually starts diversifying (by mutation or recombination) to produce variants that differ in only one or more of the seven loci that are called single-, double- or triple-locus variants (SLV, DLV and TLV). In this way, a cluster of STs in an eBURST diagram in which all STs are linked as SLVs to at least one other ST is defined as clonal complex (CC) and the founder of each CC, is defined as the most predominant ST and the one with the largest number of SLVs. Moreover, large clonal complexes typically contain subgroups and therefore have both primary and subgroup founders, where an SLV of the primary founder may have increased in frequency and diversified to generate a number of its own SLVs, thus becoming a subgroup founder (59). In the case of *S. aureus,* a CC was defined as a cluster of isolates that share at least five identical alleles (42).

The eBURST analysis can be represented graphically (see Figure 1), where each ST is represented as a circle and the frequency of each ST (i.e., the number of isolates of the ST in the input data) is indicated by the area of the circle. SLVs are connected to the primary founder of the group by a solid line. The primary group founder is represented in blue and the subgroup founder is represented in yellow (59).

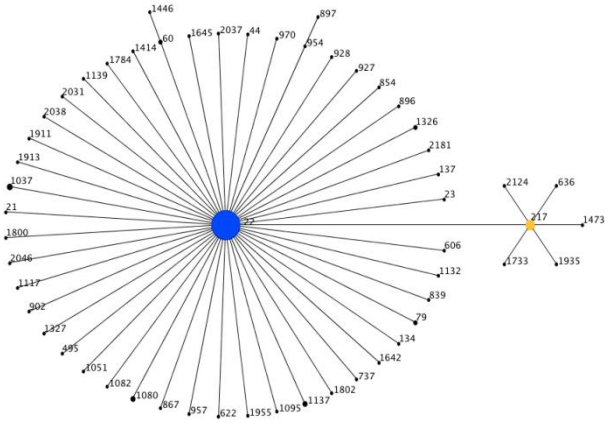

Figure 1 – eBURST diagram representing the *S. aureus* clonal complex (CC) 22. Accessed on January 5, 2012.

MLST has the big advantage of being suitable for long-term epidemiological studies, since it is based on variations in housekeeping genes that evolve slowly, allowing for an overall view of the long-term evolution and population biology of *S. aureus.* Because the data obtained from MLST analysis is based on DNA sequences it is unambiguous and can be easily interchanged. On the other hand, MLST has the disadvantages of being expensive, laborious and time consuming (44, 176).

1.7.4 SCC*mec* typing

Due to the high diversity in SCC*mec* organization and genetic content, several typing methods have been described over the years to determine the structure of this genetic element. The determination of the SCC*mec* type is epidemiologically and clinically relevant, since the currently accepted definition of MRSA clones is based on the combination of SCC*mec* type with clonal background (ST) (55).

Most of the methods described in the last years are PCR-based methods that, either by conventional PCR or multiplex PCR, rely on the amplification of different *loci* within the several types of the cassette. Basically, there are two main approaches for SCC*mec* type discrimination: one that is based on the individual determination of *mec* complex and *ccr* complex (31, 81, 83, 139), and the other that is based on the determination of the structural differences in J regions of each SCC*mec* type (22, 96, 122, 141, 206). In 2006, two studies described a new approach for the inference of the SCC*mec* type I to IV and VI, based on the sequencing of an internal fragment of the *ccrB* locus using a

pair of degenerated primers (103, 143). The sequences can then be submitted and analyzed in an online database (www.ccrbtyping.net), where a *ccrB* allele is assigned. *ccrB* showed to have a good correlation with SCC*mec* types and could be used as a first-line assay or simply as a confirmation tool of the SCC*mec* type assignment (144).
Additionally, due to the increasing number of MRSA infections in the community, the typing and subtyping of SCC*mec* type IV became important. A typing scheme was proposed by Milheiriço *et al*., based on the detection of variations within the J1 region, enabling the identification of SCC*mec* types IVa to IVh (121).
In parallel to PCR-based methods, real-time PCR strategies have also been described for SCC*mec* typing. These methods have the advantages of requiring a short time and less labor in the PCR preparation and to have a fast and easy to interpret output. Still, real-time PCR is expensive, as it requires special equipments and reagents (185).
Recently, rules were established for the nomenclature, description and identification of SCC*mec* and other SCC elements in order to avoid the ambiguities and inconsistencies in the published literature (85).
The continuous emergence of new SCC*mec* types, like the recently described SCC*mec* types IX, X and XI (63, 102), is a challenging issue for the typing strategies since the description of a new SCC*mec* type easily leads to obsolete schemes.

1.8 Molecular epidemiology of *S. aureus*

Shortly after the introduction of methicillin into clinical use to treat infections caused by penicillin-resistant *S. aureus*, the first MRSA strain was reported from UK in 1961 (86). In the following years, MRSA strains disseminated to several other European countries. However was only in the mid 1980s that MRSA became pandemic, having spread worldwide and accumulated resistance to almost all antimicrobial classes (45, 111). During this time, MRSA infections seemed to be confined mainly to hospitals and other healthcare facilities. However, in the mid-late 1990s MRSA started to emerge in the community (27, 186) and have been increasing ever since in different parts of the world. These MRSA were genetically different from those usually found in the hospitals and also in the kind of infections they caused and the populations affected (60, 132).

However, nowadays these community-associated MRSA can also be identified as a cause of hospital-onset and health-care associated infections, suggesting that certain clones have the ability to cross barriers between hospitals and the community (93, 146). Worryingly, MRSA strains that were thought to be restricted to animals are being increasingly seen also in humans, as causative agents of infection (40, 201, 202).

1.8.1 Origin and evolution of Hospital-associated MRSA (HA-MRSA)

Healthcare associated MRSA are among the most common causes of bacteremia, surgical site infections, pneumonia, and urinary tract infections in the nosocomial setting, as are also commonly associated with prosthetic-device and catheter-related infections (181). Common risk factors for HA-MRSA infections include prolonged hospitalization or residency at a nursing home, undergoing dialysis, use of indwelling catheters, Intensive Care Unit exposure, prior antimicrobial therapy or surgical procedures, and close proximity to a inpatient colonized with MRSA (30). Frequently, HA-MRSA are multidrug-resistant and carry the larger SCC*mec* elements belonging to type I, II, III, which in the particular case of SCC*mec* types II and III are known to carry additional antimicrobial resistance traits (29, 81).

According to the SENTRY Antimicrobial Surveillance Program, between 1997 and 2003, the global MRSA prevalence in hospitals was 23% in Australia, 67% in Japan, 40% in South America, 36% in USA and 23% in Europe (15, 45, 71, 182).
More recently, in Europe, the MRSA prevalence in invasive disease was 17.5% and seems to be decreasing or at least stabilizing, however with proportions ranging from 0.5 % to 52.2 %, with Portugal as the country with the highest prevalence of MRSA in healthcare-associated infections (57).
Several studies have addressed the topic of the origin of the major MRSA clones. The first theory, designated the single-clone theory, proposes that all MRSA descended from a single ancestral *S. aureus* strain that acquired SCC*mec* once (98). However this hypothesis was quickly dismissed and replaced by the multi-clone theory, according to which MRSA emerged from repeatedly acquisitions of SCC*mec* by different *S. aureus* genetic lineages and that subsequently disseminated (55).

The study wherein the first SCC*mec* acquisition was discovered was a study by Crisóstomo *et al.* (39), where MSSA and early MRSA strains isolated in 1950/1960 in Denmark and the United Kingdom (UK) were analyzed. This study showed that MSSA strains had gradually accumulated resistance determinants in parallel to antimicrobial introduction in therapeutics. Moreover it was shown that the early MRSA (ST250-I, the Archaic clone) and MSSA strains (belonging to CC8) had a similar background. Furthermore, one of the major contemporary MRSA clones (ST247-I, the Iberian clone) was found to be a SLV of ST250, suggesting it was a direct descendant of the archaic clone.

However, as Enright *et al* (55) described later, other events of acquisition occurred in genetic backgrounds not related to CC8 during *S. aureus* evolutionary history. By studying a wide collection of MRSA and MSSA strains, isolated in 20 countries between 1961 and 1999, by MLST and SCC*mec* typing, these authors demonstrated that the major MRSA clones could be grouped into five CCs: CC5, 8, 22, 30 and 45. Moreover, according to their data SCC*mec* elements appear to have been acquired by *S. aureus* strains with a distinct genetic background at least 20 times as suggested by the presence of different SCC*mec* elements within the same ST (157). More recently, a study by Nubel *et al.* (136) proposed that the SCC*mec* element was imported much more frequently than previously suggested, and that MRSA apparently emerged locally in multiple occasions. Moreover, the same authors suggest that MRSA geographical dispersal was limited, however this conclusion is not consensual.

Additionally, Enright *et al.* (55) proposed a pattern of descent within the major MRSA genetic backgrounds, confirming the findings of Crisóstomo *et al.* (39) according to which ST250-MSSA, a successful MSSA lineage of CC8, was the ancestor of the first MRSA, ST250-I (Archaic clone), as well as of other major nosocomial MRSA pandemic lineages such as ST8-IV, ST247-I (Iberian clone) and also ST239-III (Brazilian/Hungarian clone). Other major international EMRSA clones such as ST5-II (New York/Japan clone), ST22-IVh (EMRSA-15 clone), ST30-IV, ST36-II (EMRSA-16) and ST45-IV (Berlin clone) were formed within the major five CC and all derived from epidemic MSSA lineages that had acquired SCC*mec* in different occasions (55).

ST239-III is one of the most epidemic MRSA clones and also one the better studied. Hospital epidemics caused by this clone are well documented all over Europe and South America during the 1980s and 1990s and Asia and Middle East in the last decade. ST239 was the first bacterial hybrid to be identified in nature. Its chromosome appears to have evolved from the homologous recombination of a 557-kb fragment of the ST30 chromosome with the chromosome of a ST8 strain, representing a distinct branch of CC8 (156). A high genetic diversity has been associated to ST239-III, namely a high number of different PFGE macrorestriction patterns have been found associated to this clone (38, 119). Genetic diversity was also apparent when isolates of ST239-III clone from different time periods and geographic locations were analyzed by whole genome sequencing. This study demonstrated that ST239 evolved vary rapidly (1 SNP each 6 weeks), and could be further discriminated into three clades that seem to have evolved within a specific geographic region (Europe, South America and Asia). However, events of intercontinental spread were also observed to have occurred. Interestingly in Portugal both events appear to have taken place. The Portuguese and the Brazilian clones, two clones known to have dominated in Portuguese hospitals during the 1990s, both belonging to ST239 but that could be distinguished by PFGE, seem to have different origins. While the Brazilian clone most probably resulted from the introduction of a South American variant in Portugal, the Portuguese clone probably was created in Portugal or elsewhere in Europe (77).

In Table 3 are represented the major healthcare-associated MRSA clonal lineages currently spread worldwide.

Table 3 – Major HA-MRSA clones currently disseminated worldwide.

HA-MRSA clone	ST	CC	SCC*mec*
New York/Japan	5	5	II
Pediatric	5	5	IV/VI
Iberian	247	8	I
Brazilian/Hungarian	239	8	III
EMRSA-15	22	22	IV
EMRSA-16	36	30	II
Berlin	45	45	IV

Although MRSA are usually very clonal, their epidemiology has been shown to be highly dynamic. In the same geographic region, or single hospitals, it has been frequently observed that the prevalent HA-MRSA changes over time. This was the case in Hungary where, between 1994 and 2004, ST239-III was replaced by both ST5-II and ST228-I; or in Portugal were several clonal waves were documented over a 16-year period (4, 34).

Particularly in Portugal, from 1990 through 2006, several important epidemiological events took place in the Portuguese hospital with the replacement of the Portuguese clone (ST239-III variant) by the Iberian clone (ST247-IA) in 1992-1993; the emergence of the Brazilian clone in 1992 (ST239-III/IIIA) and its rapid dissemination in the following years, becoming the dominant clone between 1996 and 2000 (5). Subsequently, in 2001, the Brazilian clone was massively replaced by the EMRSA-15 clone (ST22-IVh), which became widespread in numerous Portuguese hospitals (4, 11), being the major clone in several of them. Furthermore, since its first report in Portugal as a single isolate, in 2005 (11), the New York/Japan clone (ST5-II) seemed to gain a foothold in Portuguese hospitals, representing the second major clone encountered in 2006 (4), and being suggested as the possible new leading clone in a near future.

1.8.2 The emergence and epidemiology of community-associated MRSA (CA-MRSA)

After the global spread of MRSA in the nosocomial setting, a change in the MRSA epidemiology occurred in the mid- and late 1990s. The MRSA, once confined to the hospitals, began to cause infections in the community in otherwise healthy people (27, 41, 68, 79).

Community-acquired or community associated MRSA (CA-MRSA) infections were first reported in Aboriginal patients in remote communities from Western Australia in 1993 (186). However, CA-MRSA were only recognized as a new public health threat when four healthy children died from sepsis and necrotizing pneumonia in the USA in 1998 (27).

According to the Center for Disease Control and Prevention (CDC), MRSA strains isolated in an outpatient setting or from patients within 48 hours of hospital admission

should be considered CA-MRSA. Furthermore, these patients must have no medical history of MRSA infection or colonization (<1 year from infection), no recent hospitalization (e.g. surgery), admission to a nursing home or dialysis and the patient should not have permanent indwelling devices, such as catheters or other medical invasive devices (http://www.cdc.gov/mrsa/diagnosis/index.html). However, several other definitions of CA-MRSA have been published, mostly based on the timing of the isolation of MRSA relatively to the time of admission, with or without assessment of healthcare–associated risk factors; or by the absence of risk factors for healthcare associated MRSA (132, 162).

The type of population affected by CA-MRSA and the clinical syndromes presented are different when compared to HA-MRSA. Some groups seem to be at a higher risk for CA-MRSA infections, namely children and young people, residents of inner city neighborhoods, Native American (NA) and Pacific Islander populations, incarcerated and military populations, athletes, people of low socioeconomic status, men who have sex with men, HIV patients, injecting and intranasal drug users, veterinarians, livestock handlers and pet owners (41). CA-MRSA has been mostly associated with skin and soft tissue infections although it can cause more serious infections as necrotizing pneumonia, necrotizing fasciitis, severe sepsis, folliculitis, cellulitis, impetigo, pyomyositis and myositis, septic arthritis, osteomyelitis, sepsis, and endocarditis (41).

CA-MRSA differs both genetically and phenotypically from HA-MRSA. Frequently, CA-MRSA isolates carry smaller SCC*mec* elements, usually type IV, V and VII and are resistant to fewer non-ß-lactam classes of antimicrobials. Also, CA-MRSA clones have been strongly associated with PVL, which is thought to contribute to the pathogenesis of these clones. Besides PVL, other virulence factors like ACME, phenol-soluble modulins (PSMs) and α-toxin may be relevant in CA-MRSA infections (48-50, 101, 147, 196).

In the beginning of the emergence of CA-MRSA, each genetic background of PVL-positive CA-MRSA clones that were disseminated worldwide could be associated to a specific continent, i.e., ST1 and ST8 clones in the USA, the ST30 clone in Australia and the ST80 in Europe (192).

Nowadays, CA-MRSA clones are not restrained to a specific geographic region anymore and five major PVL-positive CA-MRSA clones have disseminated worldwide:

ST1-IV clone (Asia, Europe and the USA), ST8-IV clone (Europe and the USA), ST30-IV clone (Australia, Europe and South America), ST59-V clone (Asia and the USA) and the ST80-IV clone (Asia, Europe and the Middle-East).

Additionally, some minor CA-MRSA clones have been described recently such as ST5-IV, the ST22-IV clone, the ST37-IV clone, the ST93-IV, the ST377-V and the ST766-IV.

Curiously, in the particular case of USA, a country wherein CA-MRSA account for 59% of SSTI caused by *S. aureus*, more than 95% of these infections are due to a single clone, the USA 300 clone (ST8-IVa) (177). In contrast, CA-MRSA in Europe seem to be much more diverse. Not only the USA300 clone (now emerging in this continent) but other clones like the European (ST80-IVc), the Taiwan (ST59-V) and the Southwest Pacific clone (ST30-IVc) are causes of infection in the community setting (158). The reason why Europe has a wider variety of clonal types remains unclear and needs to be clarified.

Noteworthy, there has been an increasing number of reports of healthcare-associated infections caused by CA-MRSA clones and MDR CA-MRSA were already reported (46, 124), suggesting that CA-MRSA may have already gained a foothold into the nosocomial setting (93, 146, 166).

In the table below are represented the major CA-MRSA clones disseminated worldwide:

Table 4 – Major CA-MRSA clones currently disseminated worldwide.

CA-MRSA clone	ST	CC	SCC*mec*
USA300	8	8	IV
European	80	80	IV
Taiwan	59	59	V
Southwest Pacific	30	30	IV
USA400	1	1	IV

In Portugal, a country with a high prevalence of MRSA in hospitals, seems to a have a low prevalence of MRSA causing infection in the community setting (5%). Nevertheless, some of the major CA-MRSA clones described worldwide have already

been found such as USA300, ST80 and ST398 or less disseminated clones like ST121 and ST22. Moreover, multidrug-resistant (MDR) CA-MRSA isolates were already reported in Portugal (178).

Still, it is not quite understood how CA-MRSA have emerged, if SCC*mec* has been acquired by MSSA in the community or if CA-MRSA is derived from HA-MRSA, with some studies supporting the two hypothesis (6, 128, 139).

1.8.3 Livestock Associated-MRSA (LA-MRSA)

More recently, a distinct MRSA clone, unrelated to health-care associated and community associated MRSA, has been increasingly observed among colonization in pigs and in infection in humans, namely pig farmers. This MRSA clone (ST398-IV, ST398-V), first identified in the Netherlands in 2003, is characterized by not being typeable by PFGE when cut with SmaI, due to the presence of a novel DNA methylation enzyme (16, 191).

This clone has been described in several other countries in Europe, including Denmark, Germany, UK and Portugal and in North America (Canada and USA) (73, 92, 109, 117, 154, 173). Some PVL-positive ST398 have already been found in human infection in China (203) and Europe (188, 197), which poses a serious public health problem.

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

Several studies have showed the importance of MSSA as a cause of invasive disease and that the epidemiological characteristics of infection are comparable to those of MRSA infections, namely CA-MRSA infections (123, 129, 148, 184).

In a recent survey, on the distribution of *S. aureus* causing invasive disease in Europe between September 2006 and February 2007 revealed that, compared to MRSA, MSSA are genetically more diverse and more geographically dispersed (70).

Furthermore, PVL has been increasingly found associated with some MSSA lineages, namely ST121 (128, 155). In a recent study, evaluating the global distribution and evolution of PVL among MSSA, it has been shown that most of the predominant CA-MRSA clones share the same genetic background as PVL-positive MSSA lineages.

Also, PVL-positive MSSA are disseminated worldwide, perhaps having started to gradually replace the other MSSA lineages unrelated to CA-MRSA in the last two decades. These findings suggest that PVL-positive MSSA lineages may act as a reservoir for the emergence of CA-MRSA clones (155).

Among the MSSA clonal lineages disseminated worldwide, some appear to share the same genetic background with well-known MRSA clones such as CC5, CC8, CC22, CC30 and CC45 (67, 72, 74, 137), whereas others have genetic backgrounds only observed among the MSSA population such as CC7, ST9, CC12, ST15, CC25, CC51, and CC101 (3, 72, 99). This may be explained by the fact that some genetic backgrounds seem to be more favorable to the acquisition and maintenance of *mecA* gene than others (90).

In Portugal, a study from 2005 (3) showed that the MSSA lineages found both in hospital and in the community, the ST30, ST34 and ST45 lineages, shared the same genetic background of some international MRSA pandemic clones, such as the EMRSA-16, New York/Japan, Pediatric and Berlin clones, but that at that time still had not been detected in Portugal. The exception was ST5 that shared the same genetic background with the MRSA Pediatric clone (ST5-IV), which had been already reported in Portugal (161).

1.9 Aims of the Thesis

According to European Antimicrobial Resistance Surveillance Network (EARS-Net) report of 2010, Portugal presents one of the highest MRSA prevalence (52.2%) in nosocomial infections in Europe.

In Portugal at least three clonal replacement events occurred over a 16-year period (4), suggesting that continuous surveillance is essential to be able to act on infection control in an informed manner. In Hospital Santa Maria, a major central hospital in Portugal, the frequency of MRSA has sharply increased from 30% in 1993 to 49% in 2010, but the reasons lying behind this increase are not known.

On the other hand the epidemiology of MRSA in the community in Portugal is not well known. A few episodes of infection caused by community-associated MRSA have been reported and according to a recent study by Tavares *et al.*, the frequency of infection caused by CA-MRSA isolates at hospital entrance is relatively low (5%) (178). However, the frequency of MRSA causing SSTI, the type of infection most commonly associated to CA-MRSA, among patients attending healthcare centers in Portugal, was never assessed before.

In this study we aimed to understand the epidemiological phenomenon that could explain the increase in MRSA frequency in Hospital Santa Maria within a 17-year period, through the molecular characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates collected in 1993 and 2010 using state-of-the-art typing techniques.

Moreover, we aimed to determine the frequency and clonal nature of MRSA and MSSA causing SSTI in patients attending healthcare centers in Portugal, by isolating and characterizing at the molecular level *S. aureus* from SSTI samples collected in nine different health-care centers ("Médicos Sentinela" Network).

Finally we expect to reveal the possible epidemiological links between hospital and community by comparing the population structure of MRSA in the two settings in equivalent time periods (2010/2011).

Chapter 2. Material and Methods

2.1 Bacterial collections

For the elaboration of this master thesis three distinct collections of *Staphylococcus aureus* were studied.

Two collections were assembled from the hospital setting in different time periods, consisting only of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

The third collection was assembled from the community setting through the "Médicos Sentinela" surveillance network (MS). The collection was constituted by both methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) recovered from skin and soft tissue infections.

2.1.1 Hospital Setting

Hospital Characteristics: Hospital de Santa Maria is a 1,300-bed tertiary teaching hospital integrated in CHLN (Centro Hospitalar Lisboa Norte), providing direct healthcare to approximately 350,000 people from the areas of Alvalade, Benfica, Loures, Lumiar, Odivelas and Pontinha in Lisbon, but also providing services at a regional and national level.

Bacterial collections: Two MRSA collections were assembled at Hospital de Santa Maria in two different periods, seventeen years apart. One collection included 54 MRSA isolates from inpatients, collected between January and March 1993 from pus (50%), blood (22.2%), urine (9.3%), bronchial aspirates (7.4%), sputum (5.6%), catheter (3.7%) and pleural fluid (1.8%) (38). A second collection included 520 MRSA isolates from inpatients, recovered between January and December 2010 from different sources (biopsy, vascular catheter, sputum, nasal exudate, eye exudate, blood culture, ascitic fluid, bronchoalveolar lavage, synovial fluid, pus, bronchial secretions and urine). All clinical and demographic data were recorded and provided in an Excel file.

In this study all the MRSA isolates collected in 1993 and a subset of 180 isolates collected in 2010 (out of the 520 isolates) were analyzed. The group of 180 isolates was selected according to the following criteria: the first fifteen isolates collected in each

month, recovered from blood culture, pus and urine. One isolate presented different morphologies during processing and storage, which resulted in the duplication of HSM526 into HSM526A and HSM526B. From the 181 isolates, 118 (65.2%) were from pus; 50 (27.6%) were from blood and 13 (7.2%) were from urine.

MRSA isolation and identification: *S. aureus* were isolated at the hospital microbiology laboratory using standard procedures and identified as MRSA using the automated system VITEK2 *Advanced Expert System* (BioMérieux, Marcy, L'Étoile, France).

2.1.2 Community Setting

"Médicos Sentinela" surveillance network: Médicos Sentinela network is a health surveillance system created in late 1980s, constituted by general practitioners at healthcare centers scattered throughout Portugal.

Study design: Nine healthcare centers were enrolled in this study: Centro de Saúde (CS) Águeda, CS Aveiro, CS Baltar, CS Braga, CS Cantanhede, CS Lagos, CS Mealhada, CS Soure and CS Vila Real. The samples were recovered during one year-period (between November 2010 and October 2011). The samples recovery was performed by the "Médicos Sentinela" clinician with a sterile swab and transported within a weak time period to the laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológia (ITQB) in Amies medium supplemented with charcoal PS and Viscose (Deltalab, S.L.U, Barcelona, Spain). For each sample, a questionnaire assessing risk factors for hospital contact was filled by the clinician and delivered with the sample (see Annex 5). All clinical and demographic data were recorded in an Excel file.

Bacterial collection: In this master thesis, 73 swabs collected between November 2010 and April 2011 were analyzed. In the collection, around 63% of the patients were male with a mean age of 68 years old, ranging between 16 to 93 years old.

Community-Associated MRSA definition: according to the CDC (Centers for Disease Control and Prevention) criteria, CA-MRSA infections are defined as an MRSA infection in an individual who has had: diagnosis of MRSA made in the outpatient setting or by a culture positive for MRSA within 48 hours after admission to the hospital; no history of hospitalization, surgery, dialysis, or residence in a long-term care

facility within 1 year of the MRSA culture date; no permanent indwelling catheter or percutaneous medical device and no known prior positive culture for MRSA.

Since there was a lack of information in some fields of the questionnaires assessing the fulfillment of these criteria, namely the field assessing a previous colonization/infection by MRSA (55% of the cases with no information), the term of CA-MRSA could not be directly applied and was substituted by the term CO-MRSA, standing for community-onset MRSA, as proposed by Salgado *et al.* (162). Furthermore, CO-MRSA was divided in CO-MRSA of patients with healthcare-associated risk factors and CO-MRSA of patients without healthcare- associated risk factors.

2.2 Samples processing and storage

2.2.1 Hospital de Santa Maria (HSM) collection

All isolates were previously identified as MRSA by HSM microbiology laboratory, conserved in Tryptic Soy Broth (TSB, Bacto™, BBL, Becton Dickinson, Sparks, MD, USA) (see Annex 1) with 15% glycerol, and posteriorly transported and stored at ITQB as *oc* – original culture. These *oc* cultures were inoculated on Tryptic Soy Agar (TSA, Difco™, BBL, Becton Dickinson, Sparks, MD, USA) (see Annex 1) and incubated at 37ºC for at least 16 hours, in order to obtain isolated colonies. If the culture was pure, a single colony was selected and plated again on TSA at 37ºC for 16 hours. If the culture presented colonies with distinct morphologies, each type of colony was streaked on TSA until a pure culture was obtained. The purified bacterial cultures were conserved in TSB with 15% glycerol at -80ºC and denominated as *d* - duplicate, and used for further analysis. Additionally, clumping factor and protein A detection was tested in all isolates by the latex slide agglutination test Staphytect Plus (Oxoid, Basingstoke, Hampshire, England) and the *d* culture was inoculated in a Mannitol Salt Agar plate (MSA, BBL™, Becton Dickinson, Sparks, MD, USA) (see Annex 1) for mannitol fermentation confirmation.

2.2.2 Médicos Sentinela (MS) collection

The swabs received from each healthcare center were inoculated on Mannitol Salt Agar (MSA, BBL^{TM}, Becton Dickinson, Sparks, MD, USA) and incubated at 37ºC for 48 hours. MSA plates were inspected for the medium color change (from red to yellow) and colony morphology. All different colony morphologies were picked, plated on TSA and incubated at 37ºC for at least 16 hours. These cultures were considered the *oc* – original culture. From the *oc,* a single colony was selected, plated on TSA and incubated at 37ºC for at least 16 hours, until a purified culture was obtained. These cultures were considered the *d* – duplicate. Both *oc* and *d* were conserved in TSB plus 15% glycerol at -80ºC. Additionally, clumping factor and protein A production was tested by the latex slide agglutination test Staphytect Plus (Oxoid, Basingstoke, Hampshire, England) and the *d* culture was inoculated on a MSA plate.

Isolates that fermented mannitol and produced clumping factor and protein A were included in the study. When the latex slide agglutination test produced inconclusive results, the production of coagulase was tested using the BD BBL Coagulase plasma, Rabbit test (Becton Dickinson, Sparks, Maryland, USA).

From a total of 73 swabs collected between November 2010 and April 2011, 40 *S. aureus* were isolated and included in this study.

2.3 Phenotypic characterization of *Staphylococcus aureus* from the community setting

2.3.1 Antibiotic susceptibility testing

All *S. aureus* isolates from MS collection were tested for antimicrobial susceptibility trough disk diffusion method (Kirby-Bauer) according to Clinical and Laboratory Standards Institute (CLSI) guidelines from 2008. The antibiotics to which the isolates were tested were: Oxacillin (OX), Erythromycin (E), Clindamycin (DA), Linezolid (LZD), Ciprofloxacin (CIP), Quinupristin-dalfopristin (QD), Sulfamethoxazole-Trimethoprim (SXT), Penicillin (P), Tetracyclin (TE), Fusidic Acid (FD), Rifampicin (RD), Vancomycin (VA) and Gentamycin (CN). In the case of Fusidic Acid, in which

the clinical breakpoints for the antibiotic tested were not defined in CLSI, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines from 2011 were used.

Bacterial suspensions were prepared in a sterile saline solution of 0.85% NaCl and adjusted to a turbidity of 0.5 McFarland ($1x10^8$ CFU/ml). The bacterial suspensions were inoculated on Mueller-Hinton II agar (MHA, BBL™, Becton Dickinson, Sparks, MD, USA) (see Annex 1) and incubated at 37ºC for 24 hours with discs impregnated with a known antibiotic concentration (Oxoid, Basingstoke, United Kingdom). The halo formed around the disc was measured (in mm) and the isolates were considered susceptible (S), intermediate resistant (I) and resistant (R) according to CLSI guidelines (Table 5).

Table 5 – Clinical breakpoints for *S. aureus* according to CSLI 2008 guidelines.

Antimicrobial Agent	Antimicrobial Concentration	Halo diameter (mm)		
		R	I	S
Oxacillin	5μg	≤10	11-12	≥13
Erythromycin	15μg	≤13	14-22	≥23
Clindamycin	2μg	≤14	15-20	≥21
Linezolid	30μg	-	-	≥21
Ciprofloxacin	5μg	≤15	16-20	≥21
Quinupristin-Dalfopristin	15μg	≤15	16-18	≥19
Sulfamethoxazole-Trimethoprim	25μg	≤10	11-15	≥16
Penicillin	10 units	≤28	-	≥29
Tetracyclin	30μg	≤14	15-18	≥19
Fusidic Acid*	10μg	≤24	-	≥25
Rifampicin	5μg	≤16	17-19	≥20
Vancomycin	30μg	-	-	≥15
Gentamicin	10μg	≤12	13-14	≥15

*Following EUCAST guidelines 2011. Legend: R-resistant; I-intermediate-resistant; S-susceptible.

In this master thesis, *S. aureus* isolates were considered multidrug-resistant if they were resistant to three or more antimicrobial classes (other than ß-lactam antibiotics).

2.3.2 E-test

E-test (AB BioMérieux, Solna, Sweden) was performed for isolates with a halo diameter of 14 mm or less for vancomycin, in order to determine the minimal inhibitory concentration (MIC), since the strains with reduced susceptibility to vancomycin can not be differentiated from the susceptible strains by the disk diffusion method. The bacterial suspensions (0.5 McFarland) were inoculated in Mueller-Hinton agar with 2% NaCl and incubated at 37ºC for 24 hours. According to the CLSI guidelines (2008), isolates with a MIC $\leq$ 2 μg/ml were considered susceptible; isolates with a MIC between 4 and 8 μg/ml were considered intermediate-resistant and isolates with a MIC $\geq$ 16 μg/ml were considered resistant.

2.4 Molecular characterization of *Staphylococcus aureus* from hospital and community

Both collections, from the hospital and community setting, were characterized by a set of molecular typing techniques including PFGE, *spa* typing, MLST, SCC*mec* typing and subtyping.

2.4.1 DNA extraction

Chromosomal DNA was prepared using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with the following modifications: the isolates were previously plated on TSA and incubated at 37ºC overnight. Three up to four colonies were resuspended in 100 μl of 50 mM EDTA (see Annex 1) with 5 μl of 10 mg/ml lysostaphin (Ambi Products LLC, New York, USA) and 3 μl of 10 mg/ml RNase (Sigma-Aldrich, St. Louis, USA) (see Annex 2). These suspensions were incubated at 37ºC for 60 minutes and 500 μl of Nuclei Lysis

Solution were added and the cells were resuspended. The suspensions were again incubated at 80ºC for 5 minutes to lyse the cells and cooled to room temperature. 200 μl of Protein Precipitation Solution were added and the suspension vigorously mixed for 20 seconds, followed by incubation on ice for 10 minutes. The suspensions were centrifuged at 13.000 rpm for 15 minutes at 4ºC. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 μl of room temperature isopropanol and gently mixed by inversion until the DNA strands formed a visible mass. The samples were centrifuged at 13.000 rpm for 15 minutes at 4ºC. The supernatants were poured off and the tubes drained on clean absorbent paper. A volume of 600 μl of 70% ethanol was added and the tubes were gently inverted so the DNA pellet was properly washed. The DNA samples were centrifuged again at 13.000 rpm for 15 minutes at 4ºC. After the ethanol was carefully aspirated, the tubes were drained on clean absorbent paper and the pellet left to air-dry for 15 minutes (alternatively, tubes were incubated at 80ºC with the lid open for 1 min).

Finally, 100 μl of 1X TE (see Annex 3) were added to the tubes, to rehydrate the DNA, and incubated at 4ºC overnight. The DNA was subsequently stored at -20ºC.

2.4.2 Pulsed Field Gel Electrophoresis

2.4.2.1 DNA agarose disc preparation and PFGE for *S. aureus*

The protocol used for PFGE analysis was previously described by Chung *et al.* (32).

The *d* cultures were directly inoculated into 5 ml of TSB medium and incubated at 37ºC overnight with rotation (TC-7, New Brunswick scientific Co, Edison, New Jersey, USA).

A volume of 500 μl of the overnight cultures was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 13000 rpm for 2 minutes (Biofugue *pico*, Heraeus Instruments, Germany). The supernatants were discarded with vacuum and the pellets were resuspended in 500 μl of PIV (see Annex 3). The suspension was then again centrifuged and the supernatants discarded with vacuum. A volume of 200 μl of PIV was added to each tube and the pellets resuspended. A 1:200 dilution of the suspension was prepared and the optical density (620 nm) was measured on the

spectrophotometer (Ultraspec III, Pharmacia LKB, United Kingdom). PIV was added to each bacterial suspension in order to obtain a final OD of 5.0.

The agarose discs were prepared by doing a mixture of 1:1 of the bacterial suspensions (OD=5) with 1.5% (w/v) low melting agarose (SeaPlaque agarose, FCM Bioproducts, Rockland, Maine, USA), both previously incubated at 42ºC. 20 µl droplets of this mixture were deposited onto a parafilm-coated glass and covered with a microscope slide to form a disc-like shape. The discs were incubated at -20ºC for 5 minutes and left at room temperature for 10 minutes. Lysis of cells was performed by putting the discs in 1 ml of EC solution (see Annex 3), 50 µg/ml RNase A (Sigma-Aldrich, St. Louis, USA), 100 µg/ml lysozyme (Sigma-Aldrich, St. Louis, USA) and 50 µg/ml lysostaphin (Ambi Products LLC, New York, USA) and incubated at 37ºC for at least 5 hours, followed by incubation in 500 µl of ESP solution (see Annex 3) at 50ºC overnight.

The discs were washed five times in 1X TE, each time for at least 30 minutes at room temperature, and stored at 4ºC in this solution until use.

Total DNA contained in the agarose disc was restricted with 13.5U of SmaI enzyme in 1X NEB 4 buffer (New England Biolabs, Beverly, USA). The discs were incubated at 25ºC overnight.

The chromosomal DNA fragments were then separated in a 1% agarose gel (Seakem LE, Lonza, Rockland, ME, USA) in 0.5X TBE (see Annex 3). The electrophoresis was carried out in a CHEF DR III apparatus (Bio-Rad, Hercules, California, USA) with 0.5X TBE as running buffer. The Lambda Ladder PFG Marker (New England Biolabs, Beverly, USA) was used as molecular weight marker. The running conditions were the following: 6V/cm with initial pulses of 5 seconds, which increased until 35 seconds, for a total run of 23 hours. Next, the gel was stained with a 0.375 µg/ml ethidium bromide solution (Bio-Rad, Hercules, California, USA) for about 20 minutes followed by visualization and data recording under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.2.2 Analysis of PFGE macro restriction pattern

PFGE macro restriction patterns were analyzed in Bionumerics software (v4.61 Applied Maths, Saint-Martens-Latem, Belgium). Gel photographs were converted to 8-bit

grayscale TIFF images and treated for analysis using the resources of Bionumerics software. The intra and inter gels PFGE runs were normalized using *S. aureus* NCTC8325 previously loaded in each gel as a reference. Band assignments were manually curated after automatic band detection for all gel images, where bands ranging between 10kb and 674kb were considered for analysis.

The similarity of PFGE patterns within and between gels was performed with the following settings: optimization of 0.5% and position tolerance of 1.25%. PFGE types and subtypes were defined by groups formed at 80% and 95% Dice similarity cutoffs respectively, on a dendrogram constructed by the unweighted-pair group method using average linkages (UPGMA). For identification to each PFGE type a capital letter was attributed and to each subtype a numerical number was also attributed.

The groups defined at these thresholds were previously shown to approximate those defined using Tenover's criteria for visual PFGE type definition (26, 58, 115, 180).

2.4.3 *mecA* gene detection

The presence of *mecA* gene in the "Médicos Sentinela" *S. aureus* isolates was performed by PCR with the primers *mecA* P4 and *mecA* P7, as previously described (141). The PCR mixture was prepared in a volume of 50 μl, as follows: 5 ng of template DNA, 1X GoTaq Flexi PCR Buffer (Promega, Wisconsin, USA), 1.5mM $MgCl_2$ (Promega, Wisconsin, USA), 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of each primer and 1.25U of GoTaq Flexi DNA polymerase enzyme (Promega, Wisconsin, USA). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes, amplification of 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for one minute), final extension at 72ºC for 10 minutes, and kept at 16ºC in the apparatus until stored at 4ºC. Whenever unspecific bands were detected isolates were retested with a higher annealing temperature (58ºC). For all reactions *S. aureus* COL strain was used as a positive control.

PCR products (10μl) were run in a 1% agarose gel, with 0.1 μg/ml ethidium bromide, at 5 V/cm for one hour. The PCR products were visualized under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.4 *spa* typing

spa typing was performed on representative isolates of each PFGE type. The amplification of *spa* polymorphic X region was accomplished by PCR as described (2) and the primers used were 1113F and 1514R. The reaction mixture was prepared in a final volume of 50 μl as follows: 5 ng of template DNA, 1X SUPER Tth PCR buffer 1 with 1.5 mM $MgCl_2$ (HT Biotechnology, Cambridge, United Kingdom); 200 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.1 μM of each primer and 1.25 U of SUPER Tth DNA polymerase (HT Biotechnology, Cambridge, United Kingdom). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) in the following conditions: initial denaturation at 80ºC for 5 minutes, amplification in 35 cycles (denaturation at 94ºC for 45 seconds, annealing at 60ºC for 45 seconds and extension at 72ºC for one minute and 30 seconds), final extension at 72ºC for 10 minutes and kept at 16 ºC in the apparatus until stored at 4ºC. For one isolate amplification was not achieved with the conditions described above and, for that reason, primers *spa* F2 and *spa* R1 were used with the following program: initial denaturation at 94ºC for 10 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds and extension at 72ºC for 30 seconds) and final extension at 72ºC for 10 minutes (97).

The PCR products (2 μl) were run in a 1% agarose gel, with 0.1 μg/ml ethidium bromide, at 5 V/cm for 30 minutes. The PCR products were visualized under UV light in Gel-Doc XR (Bio-Rad, Hercules, California, USA) and purified by column, by single tube or a 96 well plate, according to the number of samples. The purified PCR products were then sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The obtained sequences were analyzed in a software program named Ridom StaphType (v2.2.1, Ridom GmbH, Münster, Germany). To each different repeat is given an alphanumerical code and to the combination of these repeats is assigned a specific *spa* type.

The BURP (Based Upon Repeat Pattern) algorithm (Ridom StaphType software) was applied to all *spa* typing data in order to define *spa* clonal complexes (*spa* CC). The parameters used for analysis were: exclude *spa* types shorter than 5 repeats; and *spa* types are clustered if the cost between the members in the same group is equal or less than 6 (118). Last accessed at September 27, 2011.

2.4.5 Multilocus Sequence Typing (MLST)

MLST was performed as previously described (54) in representative isolates of each *spa* type for HSM collection and in representative MRSA isolates of each *spa* type and some priority MSSA isolates (with a new *spa* type or *spa* types that were not described in literature) of MS collection. The reaction mixture was prepared in a final volume of 50 μl as follows: 5 ng of template DNA, 1X SUPER Tth PCR buffer 1 with 1.5 mM $MgCl_2$ (HT Biotechnology, Cambridge, United Kingdom); 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of each primer and 1.25 U of SUPER Tth DNA polymerase (HT Biotechnology, Cambridge, United Kingdom). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) in the following conditions: initial denaturation at 94ºC for 4 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds), final extension at 72ºC for 10 minutes and kept at 16ºC in the apparatus until stored at 4ºC. Whenever needed the annealing temperature was decreased to 50ºC in order to obtain amplification.

The PCR products (2 μl) were run in a 1% agarose gel, with 0.1 μg/ml ethidium bromide, at 5 V/cm for 30 minutes. The PCR products were then visualized and recorded under UV light in Gel-Doc XR (Bio-Rad, Hercules, California, USA), and purified by column, by single tube or a 96 well plate, according to the number of samples. The purified PCR products were sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).

The sequences were analyzed using the software SeqMan (v5.03, DNASTAR, Inc.) and submitted to the MLST database (http://saureus.mlst.net) for allele attribution. Sequence

type (ST) attribution was achieved by submitting to the database the allelic profile for each strain, containing the allele number of each gene analyzed.
The eBURST algorithm (eBURST v.3) was applied to all MLST data in order to assign MLST clonal complexes (CC) (http://eburst.mlst.net). Last accessed at September 27, 2011.

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

SCC*mec* typing was performed using different typing methods whenever it was deemed necessary.

2.4.6.1 SCC*mec* typing by multiplex PCR

SCC*mec* typing was performed for all MRSA isolates. The determination of SCC*mec* structure was performed by multiplex PCR as previously described (122). The following strains were used as controls: COL (SCC*mec* type I), N315 (SCC*mec* type II), ANS46 (SCC*mec* type III), MW2 (SCC*mec* type IVa), WIS (SCC*mec* type V) and HDE288 (SCC*mec* type VI). The PCR mixture was prepared in a final volume of 50 μl, as follows: 5 ng of template DNA, 1X AmpliTaq PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA); 160 μM dNTPs (Bioron, Ludwigshafen, Germany); 0.2 μM of primers kdp F1 and kdp R1; 0.4 μM of primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1 F, and SCC*mec* V J1 R; 0.8 μM of primers *mecI* P2, *mecI* P3, *dcs* F2, *dcs* R1, *mecA* P4, *mecA* P7, *ccrB*2 F2, *ccrB*2 R2, *ccrC* F2, and *ccrC* R2; and 1.25 U of Amplitaq DNA polymerase enzyme (Applied Biosystems, Roche, Branchburg, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 53ºC for 30 seconds and extension at 72ºC for one minute), final extension at 72ºC for 4 minutes, and kept at 16ºC in the apparatus until stored at 4ºC.
PCR products (10 μl) were resolved in a 3% agarose gel (Seakem LE, Lonza, Rockland, ME, USA) in 0.5X TBE buffer, containing 0.1 μg/ml of ethidium bromide, at 4 V/cm

for 2.5 hours (BioRad, Hercules, CA, USA). The PCR products were then visualized and recorded under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.6.2 SCC*mec* IV subtyping

All isolates carrying SCC*mec* IV were further characterized by multiplex PCR as previously described (121) to determine the SCC*mec* IV subtype. The following strains were used as controls: MW2 (SCC*mec* type IVa), 8/6-3P (SCC*mec* type Ivb), Q2314 (SCC*mec* type Ivc), JCSC4469 (SCC*mec* type Ivd), M03-68 (SCC*mec* type Ivg) and HAR22 (SCC*mec* type Ivh). The PCR mixture was prepared in a final volume of 50 μl, as follows: 5 ng of template DNA, 1X SUPER Tth PCR buffer 1 with 1.5 mM $MgCl_2$ (HT Biotechnology, Cambridge, United Kingdom); 160 μM dNTPs (Bioron, Ludwigshafen, Germany); 0.2 μM of primers J1 IVa F, J1 IVa R, J1 Ivb F and J1 Ivb R; 0.4 μM of primers *ccrB*2 F, J1 Ivc F and J1 Ivc R; 0.8 μM of primers *ccrB*2 R, J1 Ivd F and J1 Ivd R; 0.9 μM of primers J1 Ivg F and J1 Ivg R and 1.8 μM of primers J1 Ivh F and J1 Ivh R; and 1.25 U of SUPER Tth DNA polymerase (HT Biotechnology, Cambridge, United Kingdom). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes, amplification in 35 cycles (denaturation at 94ºC for 30 seconds, annealing at 48ºC for 30 seconds and extension at 72ºC for two minutes), final extension at 72ºC for 4 minutes, and kept at 16ºC in the apparatus until stored at 4ºC.
PCR products (20 μl) were resolved in a 2% agarose gel (Seakem LE, Lonza, Rockland, ME, USA) in 1X TAE buffer (see Annex 3), containing 0.1 μg/ml ethidium bromide, at 5 V/cm for one hour and visualized under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.6.3 *mec* complex class and *ccrAB* type determination

The *mec* complex class was defined by the structure of *mec* complex components (*mecA* gene and its regulatory genes, *mecI* and *mecR1*) as previously described (139). Such

approach was used whenever no SCC*mec* type could be assigned through the PCR multiplex strategy.

The control strains used were *S. aureus* ANS46 (*mec* gene complex class A) and COL (*mec* gene complex class B). The PCR mixture was prepared in a final volume of 50 μl, as follows: 5 ng of template DNA, 1X AmpliTaq PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of primers mI4 and mI3 (*mec* gene complex class A) or mA6 and IS5 (*mec* gene complex class B) and 1.25 U of Amplitaq DNA polymerase enzyme (Applied Biosystems, Roche, Branchburg, New Jersey, USA). Both amplification reactions for *mec* gene complex A and B were carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes), final extension at 72ºC for 10 minutes, and kept at 16ºC in the apparatus until stored at 4ºC.

The determination of *ccrAB* allotypes was performed by multiplex PCR as previously described (139). The following *S. aureus* strains were used as controls: COL (*ccrAB* type 1), N315 (*ccrAB* type 2) and ANS46 (*ccrAB* type 3). The PCR mixture was prepared in a final volume of 50 μl, as follows: 5 ng of template DNA, 1X AmpliTaq PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 240 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of primer α1, 0.8 μM of primers α2, α3, βc, and 1.25 U of Amplitaq DNA polymerase enzyme (Applied Biosystems, Roche, Branchburg, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 4 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes), final extension at 72ºC for 10 minutes, and kept at 16ºC in the apparatus until stored at 4ºC.

The PCR products (10 μl) were run in a 1% agarose gel in 1X TAE buffer, containing 0.1 μg/ml of ethidium bromide, at 5 V/cm for one hour (BioRad, Hercules, CA, USA). The PCR products were then visualized and recorded under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.6.4 *ccrAB*4 typing

For all the isolates that could not been assigned a *ccrAB* allotypes the screening for the presence of *ccrAB* type 4 was performed (142). The strain used as control was *S. aureus* HDE288 (*ccrAB*4; SCC*mec* VI). The PCR mixture was prepared in a final volume of 50 μl, as follows: 5 ng of template DNA, 1X AmpliTaq PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems, Roche, Branchburg, New Jersey, USA), 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of primers *ccrB*4 F and *ccrB*4 R, and 1.25 U of Amplitaq DNA polymerase enzyme (Applied Biosystems, Roche, Branchburg, New Jersey, USA). The amplification reaction was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) with the following conditions: initial denaturation at 94ºC for 10 minutes, amplification in 30 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes), final extension at 72ºC for 4 minutes, and kept at 16ºC in the apparatus until stored at 4ºC.

The PCR products (10 μl) were run in a 1% agarose gel in 1X TAE buffer, containing 0.1 μg/ml of ethidium bromide, at 5 V/cm for one hour (BioRad, Hercules, CA, USA). The PCR products were then visualized and recorded under UV light in Gel-Doc XR apparatus (Bio-Rad, Hercules, California, USA).

2.4.7 Screening of virulence factors

2.4.7.1 Detection of Panton-Valentine Leukocidin gene (PVL)

The presence of Panton-Valentine Leukocidin genes, *lukS*-PV and *lukF*-PV, was detected by PCR as described by Lina *et al.* (104). The strain used for positive control of *pvl* gene was *S. aureus* MW2 and the primers were PVL-1 and PVL-2. The PCR mixture was prepared in 50 μl of reaction mixture, as follows: 5 ng of template DNA, 1X GoTaq Flexi PCR Buffer (Promega, Wisconsin, USA), 1.5mM $MgCl_2$ (Promega, Wisconsin, USA), 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of each primer and 1.25U of GoTaq Flexi DNA polymerase enzyme (Promega, Wisconsin, USA). The amplification reaction was carried out in a PCR apparatus (Professional

Standard Thermocycler, Biometra, Ludwigshafen, Germany) in the following conditions: initial denaturation at 94ºC for 5 minutes, amplification in 25 cycles (denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for one minute), final extension at 72ºC for 7 minutes and kept at 16ºC in the apparatus until stored at 4ºC. The PCR products (10 μl) were run in a 1% agarose gel in 1X TAE buffer, containing 0.1 μg/ml ethidium bromide, at 5 V/cm for one hour (BioRad, Hercules, CA, USA). The PCR products were visualized under UV light in Gel-Doc XR (Bio-Rad, Hercules, California, USA).

2.4.7.2 Detection of arginine catabolic mobile element (ACME)

The presence of ACME was determined by the amplification of *arcA* and *opp3* genes by PCR. The primers used were *arcA* F and *arcA* R for *arcA* gene (47) and AIPS.45 and AIPS.46 for *opp3* gene (50). The strain used as positive control was a *S. aureus* USA300 strain. The reaction mixture was prepared in a final volume of 50 μl as follows: 5 ng of template DNA, 1X GoTaq Flexi PCR Buffer (Promega, Wisconsin, USA), 1.5 mM $MgCl_2$ (Promega, Wisconsin, USA), 160 μM dNTPs (Bioron, Ludwigshafen, Germany), 0.4 μM of each primer and 1.25U of GoTaq Flexi DNA polymerase enzyme (Promega, Wisconsin, USA). The amplification reaction of *arcA* gene was carried out in a PCR apparatus (Professional Standard Thermocycler, Biometra, Ludwigshafen, Germany) in the following conditions: initial denaturation at 95ºC for 4 minutes, amplification in 30 cycles (denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes), final extension at 72ºC for 10 minutes and kept at 16ºC in the apparatus until stored at 4ºC. The amplification reaction of *opp3* gene was also carried out in the following conditions: initial denaturation at 95ºC for 4 minutes, amplification in 30 cycles (denaturation at 95ºC for 1 minute, annealing at 57ºC for 1 minute and extension at 72ºC for 2 minutes), final extension at 72ºC for 10 minutes and kept at 16 ºC in the apparatus until stored at 4ºC. Whenever unspecific bands appeared, the annealing temperature was raised until 59ºC. The PCR products (10 μl) were run in a 1% agarose gel in 1X TAE buffer, containing 0.1 μg/ml ethidium bromide, at 5 V/cm for one hour (BioRad, Hercules, CA,

USA) and visualized under UV light in Gel-Doc XR (Bio-Rad, Hercules, California, USA).

2.4.8 PCR products purification and sequencing

The great majority of the PCR products were purified with the High Pure 96 UF cleanup Kit (Roche Applied Science, Mannheim, Germany) linked to a vacuum manifold, according to the manufacturer's instructions. PCR amplification products were transferred to a High Pure UF Cleanup plate and vacuum was applied until all liquid had passed through the filter membranes in the wells. A volume of 100 μl of Wash Buffer was added and vacuum was applied once again until all the Wash Buffer had passed through the membranes. A volume of 30 μl of resuspension buffer was added and the plate was incubated for 15 minutes at room temperature. The purified PCR amplification products were then transferred to a new 96 well plate and sent to capillary sequencing (Macrogen Inc., Seoul, South Korea).
When the PCR products were in low number, these were purified with High Pure PCR products Purification kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions.

2.5 MRSA clone definition

In this thesis, MRSA clones were defined by their ST and their type of SCC*mec* as proposed by Enright *et al.*(54).

Chapter 3. Results

3.1 Clonal distribution and epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) collected from inpatients in the hospital setting

3.1.1 Molecular characterization of hospital-associated MRSA isolates from the first study period (1993)

The collection of 54 MRSA isolates recovered in 1993 in Hospital de Santa Maria (HSM), was characterized by PFGE, *spa* typing, MLST and SCC*mec* typing and subtyping. During this period, two main clones were identified circulating in HSM: the Iberian clone (ST247-I) and the Portuguese clone (ST239-III var), accounting for approximately 72% (n=39) and 28% (n=15) of the isolates, respectively (see Figure 2).

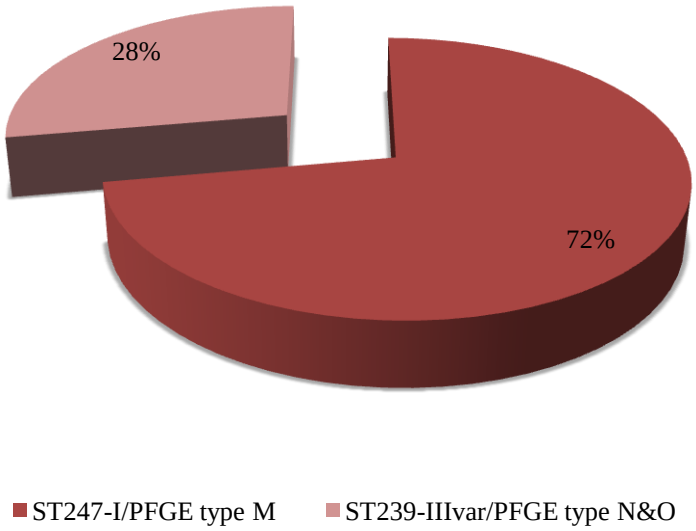

Figure 2 – MRSA population structure of isolates collected at HSM in 1993 based on MLST, SCC*mec* and PFGE type.

The Iberian clone, ST247-I, with all isolates belonging to a single PFGE type M (n=39), was characterized by *spa* types t008 and t051. Additionally, all isolates carried SCC*mec* type I, with the exception of a single isolate that was positive for two additional loci characteristic of SCC*mec* types III and V (primers RIF5 F10/RIF5 R13 and ccrC

F2/ccrC R2, respectively when multiplex PCR was used). The assignment of SCC*mec* type I in this isolate was confirmed by the presence of the *mec* complex class B and *ccrAB* type 1, similarly to the prototype strain for SCC*mec* type I (COL) that was used as positive control as assessed by individual PCR reactions (see Table 6).

The Portuguese clone, ST239-III var, was represented by isolates belonging to two different PFGE types, PFGE type N (n=12) and O (n=3), sharing a similarity of approximately 77%, and both characterized by *spa* type t421. All isolates belonging to both PFGE types carried an SCC*mec* type III variant, since no amplification could be retrieved from SCC*mec* type III J1 locus (primer RIF5 F10/RIF5 R13). The assignment of SCC*mec* type III was confirmed by the presence of *mec* complex class A and *ccrAB* type 3, similarly to prototype strain for SCC*mec* III (ANS46) that was used as positive control (see Table 6).

In order to establish the relatedness between the different isolates, the BURP algorithm was applied to *spa* typing data and *spa* clonal complexes (*spa-CC*) were assigned (see Figure 3). Both *spa* types t008 and t051 belong to the same *spa-CC*, however no founder was assumed. Concerning *spa* type t421, it was considered a singleton, not grouping in the *spa-CC* of t008 and t051 (see Table 5). Furthermore, the separation of *spa* types t008 and t051 and t421 to different *spa-CC* is in agreement with the fact that these *spa-CC* are grouped into different PFGE types.

Figure 3 – BURP analysis of *spa* types of the isolates belonging to the HSM isolates collected in 1993. One *spa*-CC (008/051) was found with no founder assigned, comprising *spa* types t008 and t051. *Spa* type t421 was considered a singleton. The black dots correspond to different *spa* types and related *spa* are linked by lines.

Similarly to *spa* algorithm BURP, MLST data can be analyzed by the eBURST algorithm to access the clonal complexes (CC) formed by related STs. MLST data were analyzed to perform a cluster analysis and a comparison with the whole *S. aureus* MLST database (http://saureus.mlst.net/). Through the eBURST algorithm, ST247 and

ST239 were both included into clonal complex 8 (CC8), in which ST8 is the predicted founder and ST239 and ST247 are single and double-locus variants of ST8, respectively.
Globally, the results show two distinct clones. Even though both clones belong to CC8, they are characterized by distinct PFGE type, *spa* types, ST and SCC*mec* types.

All isolates from the 1993 HSM collection were screened by PCR for the presence of Panton Valentine Leukocidin (PVL) and the arginine catabolic mobile element (ACME). However, none of the isolates carried the genes encoding these virulence determinants.

The main characteristics of MRSA isolates from 1993 HSM collection are listed in Table 6.

Table 6 – Main characteristics of MRSA isolates from Hospital de Santa Maria collected in 1993.

PFGE, no. isolates (%)	**SCC*mec* (no. isolates)**	***spa* type**	**ST (CC)**	**PVL**	**ACME**	**MRSA clone**
M, 39 (72.2%)	I[1], (39)	t008/ t051	247 (8)	-	-	Iberian
N, 12 (22.2%)	III variant, (12)	t421	239 (8)	-	-	Portuguese
O, 3 (5.6%)	III variant, (3)			-	-	

[1]One isolate with SCC*mec* type I variant: presence of *mec* complex class B and *ccrAB* type 1 was confirmed in simplex PCR, as described in Chapter 2.
(-) absent.

3.1.2 Molecular characterization of hospital-associated MRSA isolates from the second study period (2010)

The 181 MRSA isolates collected in HSM in 2010 were also characterized by PFGE, *spa* typing, MLST and SCC*mec* typing and subtyping.

From a global point of view, nine MRSA clones were represented in the 2010 HSM collection: ST22-IVh (EMRSA-15 clone), ST105-II (New York/Japan clone related), ST5-IVc (Pediatric clone), ST125-IVc (Pediatric clone related), ST36-II (EMRSA-16 clone), ST2246-III (Brazilian clone related), ST1-IVa (USA400 clone related), ST8-IVa and ST8-VI (see Figure 4 and Table 7).

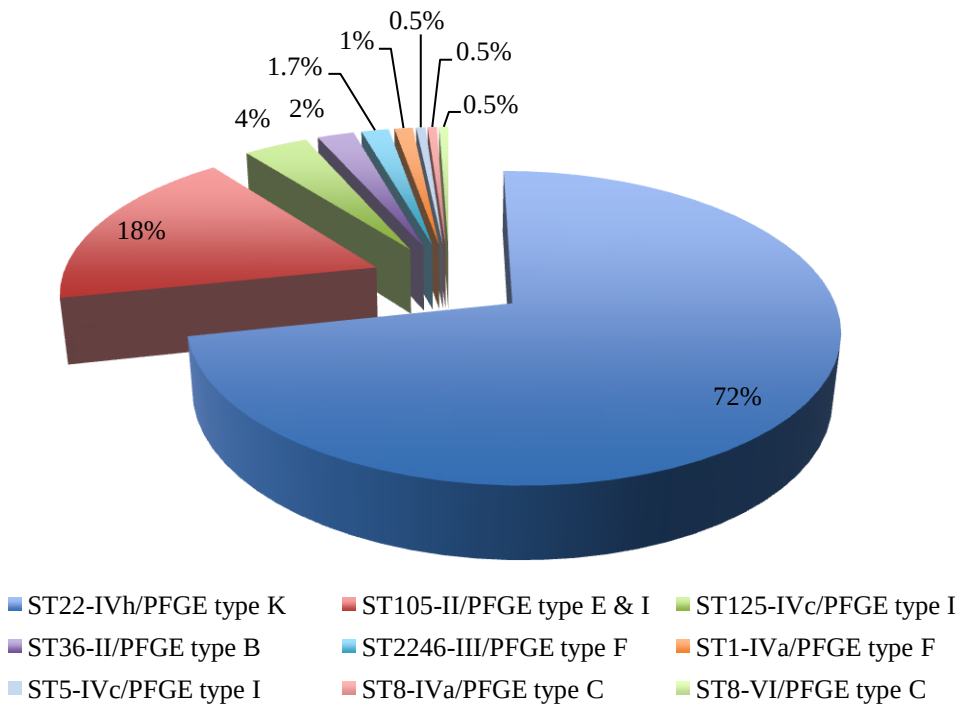

Figure 4 – MRSA population structure of isolates collected in HSM in 2010 based on MLST, SCC*mec* and PFGE type.

The major clone in the collection, ST22-IVh, the EMRSA-15 clone, accounting for 72% of the isolates, was characterized by PFGE type K and carried SCC*mec* type IVh. Additionally, as many as six different *spa* types were assigned to this clone: t2357 (n=79), t910 (n=31), t025 (n=7), t032 (n=6), t1467 (n=5) and t1302 (n=1). This clone belonged to clonal complex 22 as determined by eBURST.

We considered that the second major clonal group was a set of three highly related clones; they were related to the New York/ Japan clone (ST5-II) or to the Pediatric clone (ST5-IVc) or were single locus variants (SLV) of these clones and all belonged to CC5.

Among this group, the most prevalent clone was ST105-II, accounting for 18% of the isolates. Clone ST105-II was characterized by two PFGE types, PFGE type I (n=30) and PFGE type E (n=2), that shared a similarity of 77%, which is very close to the threshold that was considered to group isolates into the same PFGE type. Additionally this clone carried SCC*mec* type II and was characterized by *spa* type t002.

The second most prevalent clone within this group was ST125-IVc, related to the Pediatric clone, which accounted for 4% of the isolates. These isolates belonged to PFGE type I (n=7), harbored SCC*mec* type IVc and were characterized by *spa* type t067 (n=6) and t002 (n=1).

Finally in this group, we identified a single isolate of clone ST5-IVc, the Pediatric clone. This isolate was characterized by belonging to the PFGE type I, *spa* type t535 and carrying the SCC*mec* type IVc.

Overall, these three clones, ST105-II, ST125-IVc and ST5-IVc, represented approximately 22% of the entire collection.

Together, the two major groups of isolates (EMRSA-15 and the group of the three different clones related to CC5) accounted for more than 90% of the collection. The remaining 6% of the collection comprised five minor clones belonging to four different clonal complexes.

Among the minor clones, ST36-II, also known as EMRSA-16, was the most prevalent. This clone accounted for 2% of the isolates (n=4) and was characterized by PFGE type B, SCC*mec* type II, *spa* type t018 and belonged to CC30.

Another minor clone was ST2246-III, an SLV from the Brazilian clone that accounted for 1.7% of the isolates (n=3) and was characterized by PFGE type F, *spa* type t037 and carried SCC*mec* type III. This clone presented a genetic background (CC8) related to the MRSA isolates recovered in 1993 in this same hospital.

The ST1-IVa clone, related to the CA-MRSA USA400 clone, accounted for 1% (n=2) of the isolates. This clone was characterized by PFGE type F, *spa* type t127 and carried the SCC*mec* type IVa. Moreover, belonged to the CC15 clonal complex.
Surprisingly, clones ST2246-III and ST1-IVa were grouped in the same PFGE type, although they contained completely distinct *spa* types and ST. Nevertheless they could be clearly separated if a more restrictive cutoff level was considered in PFGE (95% of similarity, corresponding to PFGE subtype cut-off).

The remaining minor clones found were ST8-IVc and ST8-VI clones that were represented by single isolates each (1%). Both isolates were characterized by PFGE type C and belonged to CC8, but while ST8-IVc clone was characterized by *spa* type t008 and carried SCC*mec* type IVc, ST8-VI clone was characterized by *spa* type t024 and carried SCC*mec* type VI.

Additionally, one isolate did not amplify SCC*mec* or *mecA* gene and consequently was considered a methicillin-susceptible *S. aureus* (MSSA). This isolate was the single representative of PFGE type A and was associated with *spa* type t884, which has already been reported associated to MSSA isolates (http://spaserver.ridom.de).

Similarly to the 1993 collection, the BURP algorithm was applied to all *spa* data obtained (see Figure 5).
Four different *spa*-CC were defined among the 180 MRSA isolates analyzed, grouping 9 out of the 14 *spa* types identified. The major *spa*-CC, grouping all EMRSA-15 isolates, comprised the *spa* types t025, t032, t1302, t1467 and t2357, having t032 as the predicted founder of the cluster – *spa*-CC 032. The second largest *spa*-CC comprised *spa* types t002 and t067, but does not have a founder assigned. The third *spa*-CC comprised *spa* types t018 and t037 and the fourth comprised *spa* types t008 and t024, neither of them with a founder assigned. Regarding *spa* type t127, it was considered a singleton and was not included in any cluster. Additionally, *spa* type t535 was excluded from analysis due to the fact of being shorter than five repeats.

Overall, *spa-CC* demonstrates a good congruence with PFGE results. The grouping of t037 and t018 in the same cluster was surprising, since they apparently belong to distinct PFGE types.

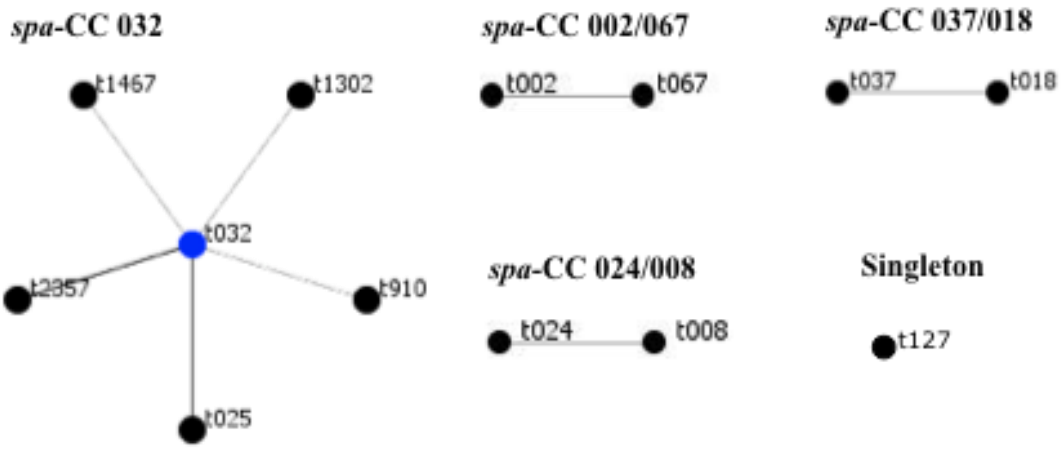

Figure 5 – BURP analysis of *spa* data obtained from isolates collected in HSM in 2010. *spa* types are represented by dots and the related *spa* types are linked by a line. The shade of the line varies according to the evolutionary cost between the *spa* types, where a darker shade represents a lower evolutionary cost. The ancestor of *spa*-CC cluster is represented in blue.

Similarly to HSM 1993 isolates, isolates from HSM 2010 collection were screened by PCR for the presence of Panton Valentine Leukocidin and ACME operon, however no positive results were obtained.

Of note, no relevant associations were found between the PFGE types/MRSA clonal backgrounds and the clinical products or the age groups in the population affected.

The main genotypic characteristics of the isolates collected in HSM in 2010 are listed in the table below (Table 7).

Table 7 – Molecular characterization of MRSA isolates from Hospital de Santa Maria collected in 2010.

PFGE type, no. isolates (%)	SCC*mec* (no. isolates)	*spa* type	ST (CC)	PVL	ACME
K, 129 (71.3%)	IVh, (129)	t025/t032/t910/t1302/ t1467/t2357	22 (22)	-	-
I, 38 (21%)	II, (30)	t002	105 (5)	-	-
	IVc, (7)	t067/t002	125 (5)		
	IVc, (1)	t535	5 (5)		
F, 5 (2.8%)	III, (3)	t037	2246 (8)	-	-
	IVa, (2)	t127	1 (15)		
B, 4 (2.2%)	II, (4)	t018	36 (30)	-	-
C, 2 (1.1%)	IVc, (1)	t008	8 (8)	-	-
	VI, (1)	t024			
E, 2 (1.1%)	II, (2)	t002	n.d	-	-

n.d - not determined; (-) absent.

3.1.3 Comparison of MRSA population structure in 1993 and 2010

The population structures of MRSA collected from inpatients in 1993 and 2010 differed almost completely and no common MRSA clones were found between the two study periods.

While in 1993 ST239-III and ST247-I accounted for all MRSA population, in 2010 the ST22-IVh and other additional seven clones were described. Overall, a highest genetic diversity was found in the MRSA collected in 2010 than in 1993, namely a high diversity was found in terms of PFGE types, SCC*mec* types, *spa* types, STs and CCs.

3.2 Clonal distribution and epidemiology of *Staphylococcus aureus* in the community – "Médicos Sentinela" surveillance network

3.2.1 *Staphylococcus aureus* in SSTI

A total of seventy-three swabs were collected from SSTI patients attending nine healthcare centers. These patients were in their majority male (63%) with a mean age of 68 years old (ranging between 16 through 93 years old).

Among the 73 swabs, 40 *S. aureus* isolates were isolated (54.8%), of which seven carried the *mecA* gene, which represents an MRSA frequency of 17.5% in the collection. The MRSA and MSSA isolates distribution by the several healthcare centers is depicted in Figure 6:

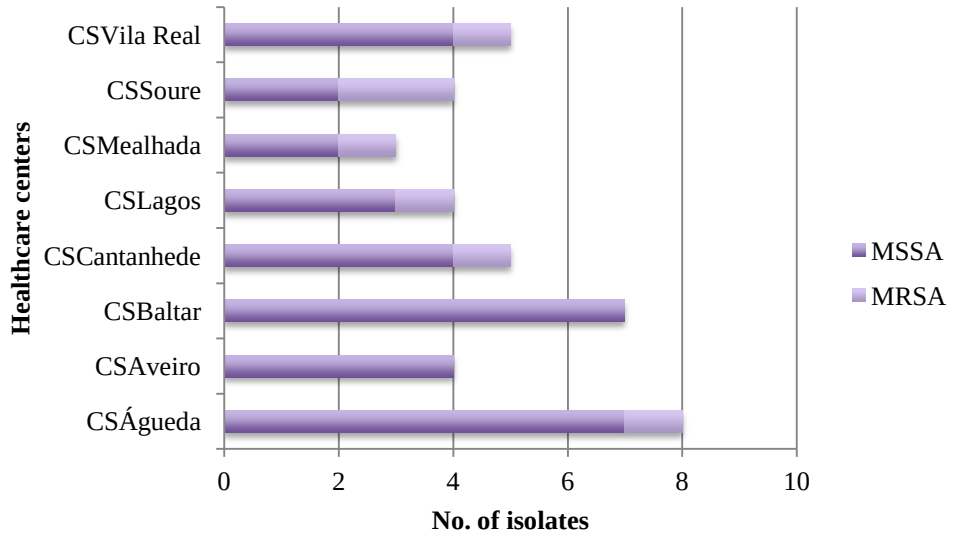

Figure 6 – MSSA and MRSA isolates distribution by the participant healthcare centers (CS – Centro de Saúde).

3.2.2 Questionnaires analysis for healthcare-associated risk factors

For each swab collected a questionnaire was filled in order to access the host risk factors. The vast majority of patients presented at least one risk factor: in the last 12 months approximately 25% of the patients had been hospitalized, at least 12.5% had contact with a long-term care facility, 15% had surgery and around 2.5% had been subjected to dialysis or had a permanent indwelling catheter. It could be confirmed that at least 10% had been previously infected/colonized by MRSA in the last 12 months. Additionally, 83% of the patients had taken antibiotics during this period (see Figure 7 and Annex 5).

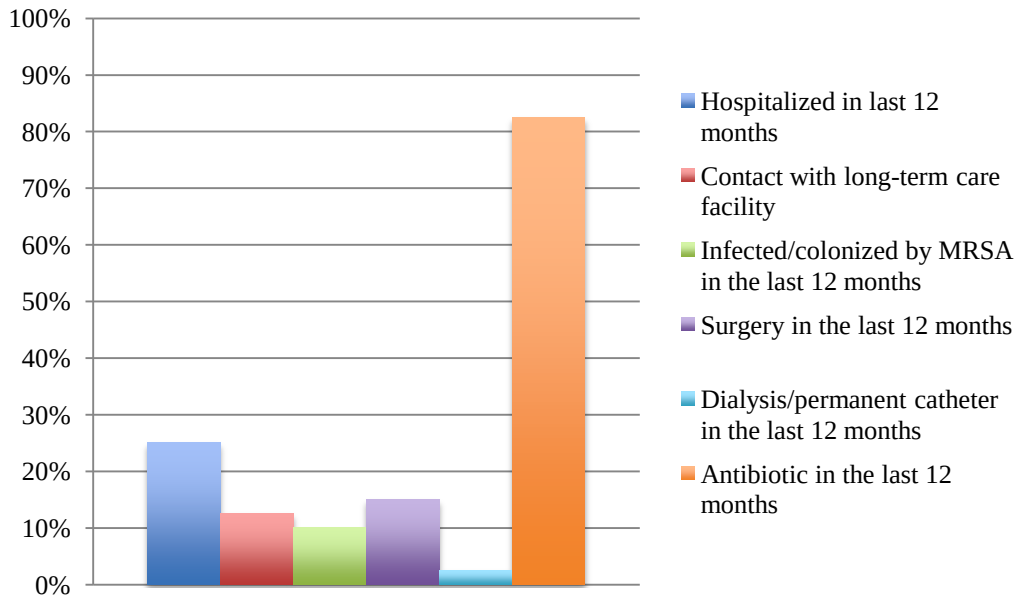

Figure 7 – Frequency of healthcare-associated risk factors assessed from the analysis of the questionnaires of the 40 *S. aureus* positive samples.

For the specific case of the seven MRSA isolates, according to the definition criteria proposed in the material and methods chapter, four isolates were considered as being community-onset MRSA without risk factors, while three were considered as community-onset MRSA with risk factors.

3.2.3 Antibiotic resistance profile

All MSSA isolates included in this study were characterized for their resistance profile against a panel of 13 antibiotics (see Figure 8). Overall, 66.7% (n=22) of the isolates were resistant to penicillin (P), 18% (n=6) were resistant to fusidic acid (FD), 12% (n=4) were resistant to gentamicin (CN), 12% (n=4) were resistant to tetracycline (TE), 12% (n=4) were resistant to ciprofloxacin (CIP), 3% (n=1) were resistant to erythromycin (E), 3% (n=1) were resistant to sulfamethoxazol-trimethoprim (SXT) and 3% (n=1) were resistant to rifampicin (RD). Additionally, one isolate (3%) presented an intermediate-resistant phenotype to oxacillin. No resistance to clindamycin (DA), linezolid (LZD), quinupristin-dalfopristin (QD) or vancomycin (VA) was observed. Around 9% of the isolates (n=3) were resistant or presented intermediate-resistance to at least 3 different antibiotic classes. Only five MSSA isolates (15%) were susceptible to all the antibiotics tested.

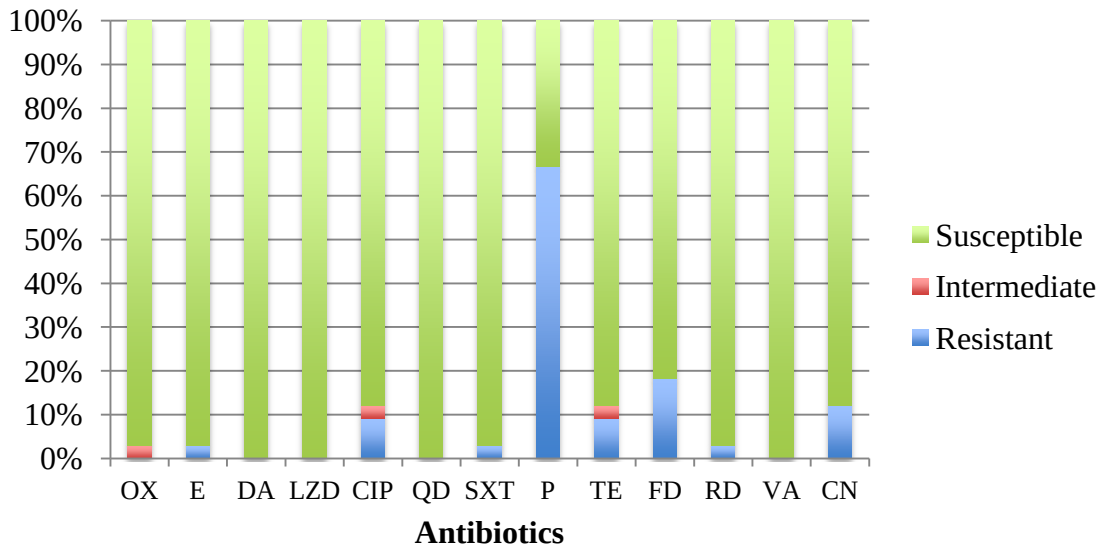

Figure 8 – Antimicrobial susceptibility of the 33 MSSA isolates collected from SSTI in healthcare centers of against a panel of 13 antibiotics. Abbreviations: S, susceptible; R, resistant; I, intermediate-resistant; OX, oxacillin; E, erythromycin; DA, clindamycin; LZD, linezolid; CIP, ciprofloxacin; QD, quinopristin-dalfopristin; SXT, trimethoprim-sulfamethoxazole; P, penicillin; TE, tetracycline; FD, fusidic acid; RD, rifampicin; VAN, vancomycin; CN, gentamicin.

The seven MRSA isolates were also tested against a panel of 13 antibiotics (see Table 8). Overall, all isolates were resistant to penicillin (P) and ciprofloxacin (CIP). Five

isolates were resistant to erythromycin (E); four were resistant to clindamycin (DA) (three of the isolates presented an inducible resistant phenotype); two were resistant to fusidic acid (FD); two were resistant to quinupristin-dalfopristin (QD). No resistance was observed to linezolid (LZD), sulfamethoxazol-trimethoprim (SXT), tetracycline (TE), rifampicin (RD), vancomycin (VA) or gentamicin (CN). In all isolates oxacillin resistance expression was associated to the carriage of the *mecA* gene, but interestingly, one isolate carrying the *mecA* gene presented a susceptible phenotype to oxacillin.

Although there was no single pattern of antibiotic resistance among MRSA isolates, these were in their majority resistant to more antibiotics classes than MSSA isolates.
All MRSA isolates were resistant to penicillin and ciprofloxacin and the great majority (n=5) also to erythromycin. The only isolates resistant to clindamycin and quinupristin-dalfopristin were also MRSA. Overall, five MRSA isolates were considered multidrug-resistant.

Table 8 – Resistance profile of the seven MRSA isolates collected from SSTI in healthcare centers of MS surveillance network.

Strain Code	OX	E	DA	LZD	CIP	QD	SXT	P	TE	FD	RD	VA	CN
CSAg10A	R	S	S	S	R	R	S	R	S	S	S	S	S
CSC3	R	R	S	S	R	S	S	R	S	R	S	S	S
CSL3	R	R	R*	S	R	S	S	R	S	S	S	S	S
CSM4	R	R	R*	S	R	S	S	R	S	S	S	S	S
CSS4	S	S	S	S	R	S	S	R	S	R	S	S	S
CSS5	R	R	R*	S	R	S	S	R	S	S	S	S	S
CSVR8	R	R	R	S	R	I	S	R	S	S	S	S	S

Abbreviations: R-resistant; I-intermediate-resistant; S-susceptible; OX, oxacillin; E, erythromycin; DA, clindamycin; LZD, linezolid; CIP, ciprofloxacin; QD, quinopristin-dalfopristin; SXT, sulfamethoxazole-trimethoprim; P, penicillin; TE, tetracycline; FD, fusidic acid; RD, rifampicin; VAN, vancomycin; CN, gentamicin.
*Inducible resistant phenotype.

3.2.4 Molecular characterization of *S. aureus* isolates collected from SSTI from patients attending healthcare centers of "Médicos Sentinela" surveillance network

All *S. aureus* isolates were characterized by PFGE, *spa* typing, SCC*mec* typing and subtyping. Additionally, MLST was performed for all the MRSA isolates and selected MSSA isolates.

3.2.4.1 Molecular characterization of MRSA isolates

The MRSA isolates belonged to three different clones: the ST22-IVh (EMRSA-15 clone), the ST5-IVc (Pediatric clone) and the ST105-II (New York/Japan clone related). Interestingly, all these three clones have also been described associated to the hospital setting (HSM collection) previously in this master thesis (see Table 9).
The majority of MRSA isolates (n=4) belonged to CC22, being characterized by PFGE type MD, SCC*mec* type IVh, *spa* types t032 and 2357 and were assigned to ST22.
The remaining MRSA isolates (n=3) belonged to CC5 and were characterized by PFGE MK. Two of these isolates were characterized by SCC*mec* type IVc, *spa* type t535 and belonged to ST5. The other isolate was characterized by SCC*mec* type II, *spa* type t002 and belonged to ST105.

3.2.4.2 Molecular characterization of MSSA isolates

Regarding the MSSA isolates, the genetic diversity presented was higher than among MRSA isolates. The ST assignments in MSSA isolates was performed based on *spa* typing data, SpaServer database information (http://spaserver.ridom.de) and published literature. For the cases in which this association could not be established, or raised some doubts, MLST was performed. A total of 13 STs, distributed into ten different CC, were identified among the 33 MSSA isolates from the community setting (see Figure 9 and Table 10).

The most prevalent clonal complex in MSSA was CC5 comprising nine isolates and representing 27% of the collection. Six isolates (18%) could be associated to ST5 and

were characterized PFGE type MK, *spa* type t010 or t535. Three isolates (9%) could be associated to ST72 and were characterized by PFGE type MG and *spa* type t148.
The second most prevalent clonal complex, representing 15% of the MSSA collection, was CC15 and comprised five isolates; three isolates (9%) were characterized by PFGE type MI, *spa* type t127 and ST1; one isolate (3%) was characterized by PFGE type MM, *spa* type t9124 and ST15; and one isolate (3%) was characterized by PFGE type MH, *spa* type t189 and ST188.

The remaining 19 MSSA isolates belonged to eight minor clonal complexes:
- Three isolates probably belonging to CC30 and representing 9% of the MSSA collection were characterized by PFGE type ME, *spa* type t012 or t1988 and could be associated to ST30.
- Three other isolates (9% of the MSSA collection) belonged to CC45 and were characterized by PFGE type MB, *spa* type t576 or t1646 and could be associated to ST45.
- The CC8 represented 9% of the MSSA collection and comprised three isolates that could be associated to ST8. Two isolates represented the PFGE type ML and were characterized by *spa* type t008. The other isolate was also characterized by *spa* type t008 but belonged to PFGE type MI.
- Three isolates belonging to CC7 and representing 9% of the MSSA collection were characterized by PFGE type MI, *spa* type t091 and could be associated to ST7.
- Two isolates representing 6% of the MSSA collection were associated to CC49 and were characterized by PFGE type MC, *spa* type t4049 and ST49.
- The remaining clonal complexes were represented by single isolates, representing each of these single isolates 3% of the collection. One isolate belonged to CC22 and was characterized by PFGE type MD, *spa* type t790 and was associated to ST22; one isolate probably belonged to CC20, being characterized by PFGE type MJ, *spa* type t164 and could be associated to ST20. The other isolate, belonging to CC398, could not be characterized by PFGE since no macro restriction profile could be obtained with SmaI enzyme. Such trait is characteristic of the ST398 genetic background. The characterization of this isolate revealed that it was *spa* type t4389 and MLST confirmed the ST398 assignment.

Finally, two isolates could not be associated to a specific clonal background. One isolate represented the PFGE type MF and was characterized by *spa* type t4592. The other isolate belonged to PFGE type MG and was characterized by *spa* type t870.

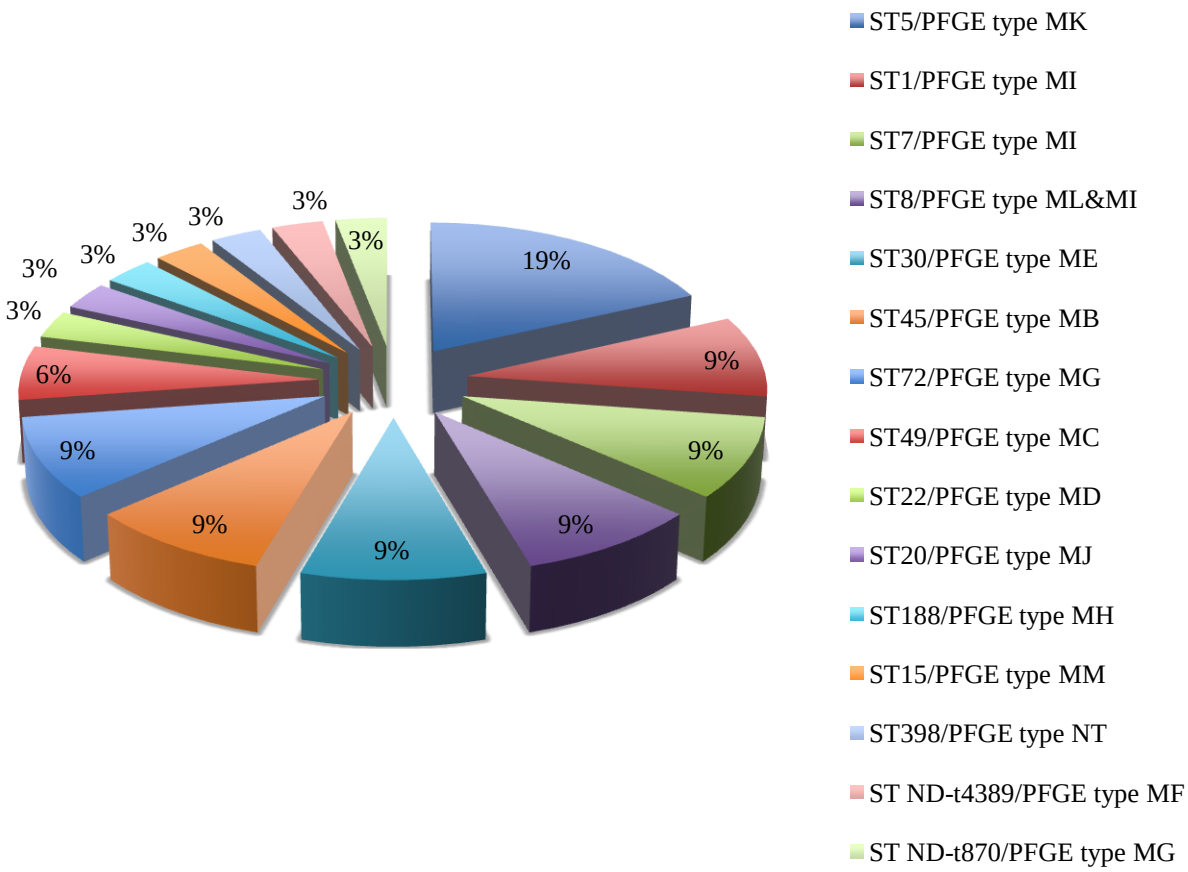

Figure 9 – MSSA population structure from isolates collected from SSTIs from patients attending the healthcare centers of MS surveillance network based on MLST and PFGE type. Legend: ST ND - Sequence type not determined.

Of note, although PFGE type MI grouped three different genetic backgrounds (CC7, CC8 and CC15), if a more restrictive analysis of PFGE type was considered (increasing the similarity between isolates) this would allow the splitting of such PFGE type in agreement to the remaining molecular characterization.

When the BURP algorithm was applied to the *spa* data obtained for both MRSA and MSSA, three *spa*-CC were obtained, while the remaining were considered as singletons. One cluster, *spa*-CC 032 comprised the *spa* types t032, t790 and t2357, being t032 the

group founder. Two additional *spa*-CC, one grouping *spa* types t002 and t010 and the other grouping *spa* types t012 and 1988, were identified, however in none of the cases a group founder was assigned. The remaining *spa* types (t008, t091, t127, t148, t164, t189, t576, t1646, t4049, t4389, t4592 and t9124) were considered singletons, while *spa* types t535 and t870 were excluded from analysis for being shorter than 5 repeats (see Figure 9). The result from BURP reinforces the associations constructed for the clonal assignment.

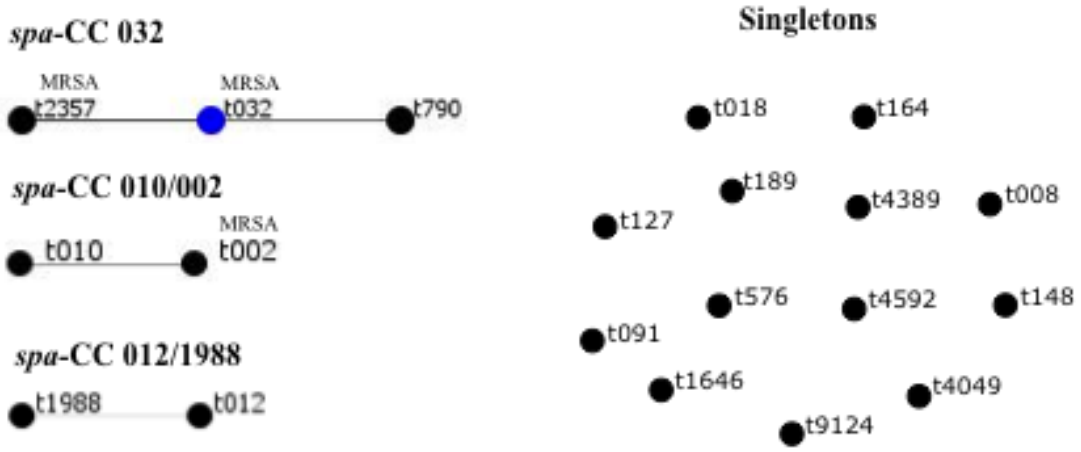

Figure 10 – BURP analysis of *spa* data obtained from isolates collected from SSTIs from patients attending healthcare centers of MS surveillance network. *spa* types are represented by dots and the related *spa* types are linked by a line. The shade of the line varies according to the evolutionary cost between the *spa* types, where a darker shade represents a lower evolutionary cost. The ancestor of *spa*-CC cluster is represented in blue.

Interestingly, when comparing the MRSA and MSSA population structures, it could be observed that some clonal backgrounds were shared between the two populations such as ST5 and ST22. Moreover, we observed that MSSA and MRSA isolates belonging to these STs, additionally, had the same (ST5-t535) or related *spa* types (ST5-t002/t010; ST22-t032/t790).

Additionally, all forty *S. aureus* isolates from "Médicos Sentinela" study collection were screened by PCR for the presence of Panton Valentine Leukocidin and ACME but none of the isolates carried the genes encoding these virulence determinants.

The main characteristics of *S. aureus* isolates from "Médicos Sentinela" collection are summarized in Table 9 and Table 10.

Table 9 – Molecular characterization of MRSA isolates from SSTIs collected from patients attending healthcare centers of MS surveillance network.

CC (%)	ST	PFGE type, no. isolates	*spa* type	SCC*mec*	PVL	ACME
22 (57%)	22	MD, 4	t032/ t2357	IVh	-	-
5 (43%)	5	MK, 2	t535	IVc	-	-
	105	MK, 1	t002	II	-	-

Table 10 – Molecular characterization of MSSA isolates from SSTIs collected from patients attending healthcare centers of MS surveillance network.

CC, (%)	ST	PFGE type, no. isolates	*spa* type	SCC*mec*	PVL	ACME
5, (27%)	ST5	MK, 6	t010/t535	-	-	-
	ST72 [a]	MG, 3	t148	-	-	-
15, (15%)	ST1 [a]	MI, 3	t127	-	-	-
	ST15	MM, 1	t9124	-	-	-
	ST188 [a]	MH, 1	t189	-	-	-
30, (9%)	ST30 [a]	ME, 3	t012/t1988	-	-	-
45 (9%)	ST45	MB, 3	t576/t1646	-	-	-
8 (9%)	ST8 [a]	ML, 2	t008	-	-	-
		MI, 1				
7 (9%)	ST7 [a]	MI, 3	t091	-	-	-
49 (6%)	ST49	MC, 2	t4049	-	-	-
20 (3%)	ST20 [a]	MJ, 1	t164	-	-	-
22 (3%)	ST22 [a]	MD, 1	t790	-	-	-
398 (3%)	ST398	NT [b], 1	t4389	-	-	-
n.d (3%)	n.d	MF, 1	t4592	-	-	-
n.d (3%)	n.d	MG, 1	t870	-	-	-

[a] clonal backgrounds were inferred based on the SpaSever (http://spaserver.ridom.de) and published literature [specific case of ST20 and ST72 (24, 179)].

[b] couldn't be restricted by SmaI.

n.d - not determined; (-) absent.

Chapter 4. Discussion and Conclusions

In accordance with the European Antimicrobial Resistance Surveillance Network (EARS-Net) report of 2010, Portugal is the European country with the highest MRSA prevalence in nosocomial invasive infection, reaching 52.2% (57). However no national strategies for MRSA surveillance and control have been successfully defined and or applied. However, previous national surveillance studies wherein single hospitals were analyzed, have allowed the description of the evolution of MRSA in different Portuguese hospitals in the last two decades. According to these studies MRSA clonal population suffered several epidemiological changes during time. Still, the factors driving clonal replacement are not understood.

Although isolated episodes of infections caused by CA-MRSA in Portugal were already reported (35, 133), these reports have been sporadic and it is not known to what extent are CA-MRSA disseminated and causing disease in the community in Portugal. Moreover the impact of the emergence of CA-MRSA in the epidemiology of MRSA in Portugal was never assessed.

In this study we described how and why MRSA clones have evolved in a Central Portuguese Hospital in Lisbon within a 17-year period. Moreover, we determined for the first time, the frequency and clonal nature of CA-MRSA causing skin and soft tissue infection at a national level. The comparison of hospital and community MRSA population structures in equivalent time periods revealed that the spillover of MRSA from the hospital, rather than the occurrence of CA-MRSA, was the main cause of SSTI in persons attending healthcare centers in Portugal.

4.1 Massive clonal replacement and diversification in Hospital de Santa Maria between 1993 and 2010

MRSA has been endemic in Hospital de Santa Maria since the mid-1980s, with an MRSA prevalence of 30% in 1993 (38). In the following years the MRSA prevalence stepped up to reach 49% in 2010, which represents an increase of more than 60%. In order to try to understand the reasons behind this great increase, two collections from HSM recovered in 1993 and 2010 were characterized and compared.

We found that all MRSA isolates collected in 1993 from infection belonged to two major clones: the Iberian clone (ST247-I), accounting for 72% of the MRSA isolates, and the Portuguese clone (ST239-IIIvariant) accounting for the remaining 28%, both belonging to clonal complex 8. This scenario is in agreement with previous reports, where the Portuguese clone was described to be the dominant clone until 1991, being later replaced by the Iberian clone that dominated between 1992 and 1998 in Portuguese hospitals (7, 163, 164).

The analysis of this same collection by PFGE years before (38) identified a total of 24 PFGE types, what clearly contrasts with the three different PFGE types identified in this study. Although the technique used in both studies was the same, the analysis was performed in totally different manners. While in the study published before the analysis of macro restriction band patterns was done visually and the Tenover criteria were used to define a PFGE type; in the study performed here the analysis was assisted by an automatic analysis software and the PFGE type was defined by a cut-off of similarity (58). Nonetheless we also could identify several different PFGE subtypes within each PFGE like was previously described.

Combining PFGE with, two additional techniques, Cla-*mecA* and Cla-Tn*554* polymorphisms, methods assessing the variability in the vicinity of *mecA* and Tn*554*, the authors were able to further discriminate this collection into 26 different clonal types. The main conclusion of this previous study was that there were an unusually high number of different *S. aureus* clones in Hospital de Santa Maria. The conclusions drawn by our study, wherein completely different methodologies were used (MLST, *spa*

typing, SCC*mec* typing) differed sharply from this. We found that only two different clonal types were present, as defined by ST-SCC*mec* combinations, but that could be further discriminated taking into consideration the results of PFGE type/subtype and *spa* type. The discrepancy of results obtained between the two characterizations can be explained by the differences in the typing methods used, namely the evolutionary time clocks of the targets analyzed and their discriminatory power, but also the tools used for analysis, in the case of PFGE, and the criteria used to define a clone.

In contrast to 1993, MRSA isolates collected from infection in 2010 belonged in its great majority (71%) to ST22-IVh, characteristic of the pandemic clone EMRSA-15, which belongs to CC22 and to three other clones (ST105-II, ST125-IVc, ST5-IVc) belonging to CC5 (22%). Additionally, four clones belonging to CC8, CC30 and CC15 were also found. The overwhelming dominance of EMRSA-15 in Hospital de Santa Maria is in accordance with previous studies, in which ST22 has been described as the most prevalent clone nowadays in Portuguese hospitals (4, 11). In these same studies, the New York/Japan clone had been suggested as the most probable next emergent MRSA clone in the nosocomial setting in Portugal. However, in this study we observed that two clones (ST105-II and ST125), belonging to CC5, other than the New York/Japan, might be candidates to become new emerging clone. These clones differing from ST5 by one point mutation on *yqiI* locus have already been described as major clones in the hospital in Switzerland (20) and Spain (13, 150, 151, 194) and as minor clones in Brazil (25), the USA (33) and Norway (61). However, as far as we are concerned, this is the first time that ST105-II and ST125-IVc are reported in a Portuguese hospital.

Since these clones accounted for 22% of the MRSA isolates in 2010, and have already proven to be capable of disseminating in the hospital and becoming dominant, it can be speculated that EMRSA-15 has started to be replaced or will be replaced by ST105-II and ST125-IVc in the near future.

Although there is no information on the MRSA population in HSM between 1994 and 2009, it could be assumed that HSM followed the national trend over the years, where

the multiresistant Brazilian clone, after its introduction in 1994-1995, rapidly disseminated and became dominant nationwide between 1998 and 2000 (7, 145). This scenario is well demonstrated in a previous study in Hospital Geral de Santo António in Oporto (10) where in the Iberian clone, the dominant clone in this hospital in the period between 1992 and 1993, was replaced by the Brazilian clone in the period of 1996-2000.

Most interestingly, three isolates related to the Brazilian clone, new SLV of ST239-III (ST2246-III), were found in the MRSA HSM collection from 2010 analyzed in this study. These could be remnants of the previous clonal wave where the Brazilian clone was prevalent or could have been imported from Asian or Middle East countries, wherein the Brazilian clone is presently the most prevalent clone.

The comparison of the results obtained for the population structure of MRSA collected in 1993 and 2010, revealed that there was an increase in the genetic diversity of MRSA clonal types. More importantly, a major shift in the clonal population of MRSA occurred within the 17-year interval. In fact, no common PFGE types, *spa* types or STs were found between the two study periods. The only CC that prevailed between the two study periods was CC8, but associated to completely distinct clones.

During its recent history, specifically during the period analyzed in this study (1993-2010), Hospital de Santa Maria has suffered several structural changes such as the integration into a Hospital center constituted also by other hospitals, that could have impacted the overall population structure of microorganisms living in the hospital, namely that of MRSA. The observed diversification of clones and increase in MRSA frequency may derive from the fact that at this moment the hospital embraces a much more diversified and larger human population.

The clonal replacement that occurred in the hospital within the 17-year study period is a characteristic of MRSA epidemiology and has occurred in the past in Portugal and all over the world. In this case, a multidrug resistant clone like the Portuguese/Brazilian clones was replaced by a clone that contains much less antibiotic resistant traits, but that appears to have an enhanced capacity to survive in multiple environments, such as the hospital (4, 8, 9, 11, 36, 87, 120), the community (126, 138) and even environmental

surfaces (170). Although the reasons lying behind the fade of one clone and the emergence of the other are not completely explained, it is believed that the newly introduced clone has a higher fitness than the established one. The fact that EMRSA-15 clone carries few resistant traits, a smaller SCC*mec* type and an increased capacity to form biofilm (172) may have conferred to this particular clone a higher mobility, growth rate and persistence capacity, what could explain its success and fitness. To further prove this hypothesis we are planning to perform fitness experiments between the several clones that were replaced during time in Portugal.

The capacity of EMRSA-15 to survive in different environments may have lead to consecutive cycles of introduction and re-introduction of the EMRSA-15 clone in the hospital through the community. A phenomenon that did not occur in the past, given the inability of the Portuguese and Iberian clones of shuffling between the two environments probably due to the carriage of bigger SCC*mec* types and multidrug resistance. This may explain the increase of the MRSA frequency observed in the hospital between 1993 and 2010.

4.2 MRSA from SSTI of persons attending health-care centers have hospital origin

One of the aims of this master thesis was to investigate the prevalence and clonal structure of *S. aureus* causing SSTIs, one of the most frequent presentations of *S. aureus* infections, especially in the community (41).
Despite the fact that Portugal has a high incidence of MRSA in the nosocomial setting, the frequency of MRSA in the nasopharinx was shown to be extremely low (less than 0.5%) (160, 179). However, the information about MRSA prevalence in infection in the Portuguese community is very scarce. Some studies conducted in Portugal wherein the frequency of CA-MRSA in infection was assessed at hospital admission showed that MRSA rate could vary between 5 and 8% (35, 178). However until the beginning of this study, the frequency of MRSA causing infection in the population attending healthcare centers in Portugal had never been evaluated.

We identified a MRSA infection rate of 17.5% among SSTI of patients attending healthcare centers. However, we cannot disregard the fact that a low number of samples were recovered. Moreover, many SSTIs are usually treated by draining without collection, what suggests that the MRSA rate could be higher than that obtained in this study.

Three out of seven MRSA were isolated from people with at least one risk factor for healthcare-associated MRSA and therefore the possibility of having been originated in the hospital cannot be discarded. All the MRSA isolates obtained in this study were recovered from elderly people, which for their intrinsic condition, have a higher probability of having a history of hospitalization and surgery or other risk factors associated with a previous contact with healthcare-associated MRSA. This situation is in clear contrast with previous studies, where MRSA infections occurring in the community in the USA seemed to affect a healthy population with no identifiable risk factors, namely children and young people (41, 79, 132).

Interestingly, the seven MRSA isolates recovered in persons attending healthcare centers belonged to clones usually associated to the nosocomial setting, such as ST5-IVc-t535, ST105-II-t002 and ST22-IVh-t032/t2357, irrespective of the fact that risk factors for hospital contact were identified or not. Noteworthy, five of these seven isolates presented a multidrug resistance profile and were exactly the same as those found among MRSA collected from Hospital de Santa Maria in 2010.
A high rate of HA-MRSA clones (ST22-IVh and ST5-II) causing infection in persons attending hospitals with no risk factors for previous hospital contact was previously observed in Portugal (178), suggesting the spill over of HA-MRSA isolates into the community. Although in our study we could not identify any CA-MRSA isolates causing SSTI, isolates belonging to CA-MRSA clones were already described as colonizers (ST82-IV, ST72-IV, ST939-IVa and ST931-VI) (179) or infectious agents (ST80-IVc, ST8-IVa, ST398-V) (178) in the community in Portugal.
Overall, the isolation of multidrug resistant nosocomial MRSA in the community suggests that these clones might be escaping from hospital into the community and

becoming established in this setting. If this is true, we might be in the verge of vast public healthcare concern in the near future.

4.3 High epidemicity of MSSA from SSTI of patients attending health-care centers

Concerning the methicillin-susceptible *S. aureus*, the isolates identified among SSTI of persons attending healthcare centers presented a higher genetic variability than their resistant counterparts. A total of 13 different STs were identified (ST1, 5, 7, 8, 15, 20, 22, 30, 45, 49, 72, 188 and 398), which included STs previously described in Portugal both in colonization and infection (ST1, 5, 8, 15, 20, 22, 30, 45, 72, 188) (3, 35, 70) and in several European countries (ST1, 5, 7, 8, 15, 20, 22, 30, 45) (70). However we also identified less frequent clones like ST49 that has been mostly associated with wild animals (squirrels) and pigs (34, 37, 46), and ST188 and ST398 described both in humans and animals (65, 91, 100, 191). The results suggest that MSSA isolates from this study, in their majority have high epidemic capacity. Moreover, we observed that some of the isolates found among SSTI in persons attending healthcare centers could have originated from animals. This might result from the fact that many of the healthcare centers analyzed were located in rural areas, where the contact with farm animals is privileged.

Interestingly, when we compared the population structures of MSSA and MRSA isolates collected from SSTI in healthcare centers, we observed that some MSSA and MRSA isolates shared the same or related genetic characteristics. This is the case of MRSA and MSSA isolates belonging to ST22 that shared PFGE type MD and had related *spa* types (t790, t032, t2357); and the case of ST5 that shared both PFGE type MK and t535. The results suggest that MRSA isolates found in the community probably lost SCC*mec* due to the absence of antibiotic pressure.

4.4 MRSA clones: evidence for the weakening of barriers between the Hospital and Community

Worldwide, there have been some reports of major nosocomial MRSA clones being isolated from the community (126) and, vice-versa, community-associated clones being found as cause of nosocomial outbreaks (93, 146, 166). However, this has been observed for few specific MRSA clones that seem to be capable of surviving and spread in both environments.

Altogether in our study we found evidence that support that the same type of phenomenon is occurring in Portugal, leading to the spread of HA-MRSA clones (ST22-IVh-t032/2357, ST105-II-t002 and ST5-IVc-t535) in the community. This might result from the high frequencies of MRSA observed inside the hospital in Portugal. However, it might also result from intrinsic characteristics of the HA-MRSA clones found nowadays in our hospitals that allowed them to spread in the community, like the carriage of SCC*mec* IV and low antimicrobial resistance profile. However, in the absence of antibiotic pressure, MRSA in the community probably lose frequently the SCC*mec* giving rise to MSSA with the same genetic background, as was the case of MSSA belonging to ST22 and ST5 clones. The role of EMRSA-15 clone in the hospital and community both in infection and colonization is well documented (4, 8, 9, 11, 36, 126, 138). And its capacity of dissemination and invasion can be even enhanced by the acquisition of additional virulence factors like ACME and PVL as was already described (105, 159, 169).

The vehicles of dissemination of HA-MRSA from the hospital into the community are not known. But EMRSA-15 clone was found as a major clone colonizing public buses handles in the region of Oporto, accounting for 91% of the isolates (170), what could function as important reservoir of this clone for the community. But patients themselves and healthcare workers may be also the main means of spreading.

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Annexes

1. Culture media

Table 1 – Culture media and composition (per Liter).

Culture media	Composition
Tryptic Soy Agar (TSA)	15g of pancreatic digest of casein; 5g of papaic digest of soybean; 5g of sodium chloride and 15g of agar. pH 7.3±0.2.
Tryptic Soy Broth (TSB)	17g of pancreatic digest of casein; 3g of papaic digest of soybean; 2.5g of dextrose; 5g of sodium chloride and 2.5g of dipotassium phosphate. pH 7.3 ± 0.2.
Mannitol Salt Agar (MSA)	5g of pancreatic digest of casein; 5g of peptic digest of animal tissue; 1g of beef extract; 75g of sodium chloride; 10g of D-mannitol; 0.025g of phenol red and 15g of agar. pH 7.4 ± 0.2.
Mueller-Hinton II Agar (MH)	2g of beef extract; 17.5g of acid hydrolysate of casein; 1.5g of starch and 17g of agar. pH 7.3 ± 0.1

2. Enzymes

Table 2 – Enzymes for cellular lysis and DNA purification.

Enzyme	Composition
RNase A	10 mg/ml in sterile purified water. Boiled at 100ºC for 15 minutes. Conserved at -20ºC in 200 µl aliquots.
Lisozyme	20 mg/ml in sterile purified water. Conserved at -20ºC in 200 µl aliquots.
Lysostaphin	10 mg/ml in sterile 20mM sodium acetate, pH 4.5. Conserved at -20ºC in 200 µl aliquots.

3. Buffers and Solutions

Table 3 – Buffers and DNA extraction solutions.

Solution	Preparation of stock solution
PIV	0.01 mM Tris-HCl pH 8.0; 1 M NaCl
Tris buffer	10 mM Tris-HCl pH 8.0.
TE 10X	10mM Tris pH 7.5; 1 mM EDTA pH 8.0.
TAE 50X	0.5 M Tris; 0.05 M EDTA pH 8.0; 1 M acetic acid.
TBE 10X	1M Tris; 1 M boric acid; 0.2 M EDTA pH 8.0. Adjust to pH 8.5.
EC lysis solution	6mM Tris pH 8.0; 1 M NaCl; 100 mM EDTA pH 8.0; 0.2% (w/v) sodium deoxycholate; 0.5% (w/v) sodium laurylsarcosine. After sterilization, enzymes were added: 50 µg/ml RNase A, 100 µg/ml lysosyme and 50 µg/ml lysostaphin.
ESP solution	0.5 M EDTA pH 9.0; 1% sarcosyl. After sterilization, 1mg/ml of proteinase K was added.

4. Primers List

Table 4 – Nucleotide sequences (5'-3' orientation) of primers used in this study. Target region and expected size of the amplicon is shown, as well the bibliographic references where the primers were first described.

Assay	Target region	Primers sequence (5' to 3')	Amplicon Size (bp)	Reference
mecA gene detection	*mecA* gene	mecA P4: TCCAGATTACAACTTCACCAGG mecA P7: CCACTTCATATCTTGTAACG	162	(141)
mec complex identification	*mec* complex A (*mecI*)	mI4: CAAGTGAATTGAAACCGCCT mI3: CAAAAGGACTGGACTGGAGTCCAAA	180	(139)
	mec complex B (IS1272-*mecA*)	IS5: AACGCCACTCATAACATATGGAA mA6: CATAACTTCCCATTCTGCAGATG	2000	(139) (88)
ccr genes identification	*ccrAB1*	α1: AACCTATATCATCAATCAGTACGT βc: ATTGCCTTGATAATAGCCITCT	700	(139)
	ccrAB2	α2: TAAAGGCATCAATGCACAAACACT	1000	
	ccrAB3	α3: AGCTCAAAAGCAAGCAATAGAAT	1600	
	ccrAB4	ccrAB4 F1: TCATCAATAAGTATGGAACG ccrAB4 R1: TTTCTTGCGACTCTCTTGG	1500	(142)

Table 4 – Continuation.

Assay	Target region	Primers sequence (5’ to 3’)	Amplicon Size (bp)	Reference
SCC*mec* typing	SCC*mec* I, J1 region	CIF2 F2: TTCGAGTTGCTGATGAAGAAGG CIF2 R2: ATTTACCACAAGGACTACCAGC	495	(141), (122)
	SCC*mec* V, ccr complex	ccrC F2: GTACTCGTTACAATGTTTGG ccrC R2: ATAATGGCTTCATGCTTACC	449	
	SCC*mec* III, J3 region	RIF5 F10: TTCTTAAGTACACGCTGAATCG RIF5 R13: ATGGAGATGAATTACAAGGG	414	
	SCC*mec* V, J1 region	SCCmec V J1 F: TTCTCCATTCTTGTTCATCC SCCmec V J1 R: AGAGACTACTGACTTAAGTGG	377	
	SCC*mec* I, II, IV and VI – J3 region	dcs F2: CATCCTATGATAGCTTGGTC dcs R1: CTAAATCATAGCCATGACCG	342	
	SCC*mec* II and IV, ccr complex	ccrB2 F2: AGTTTCTCAGAATTCGAACG ccrB2 R2: CCGATATAGAAWGGGTTAGC	311	
	SCC*mec* II, J1 region	kdp F1: AATCATCTGCCATTGGTGATGC kdp R1: CGAATGAAGTGAAAGAAAGTGG	284	
	SCC*mec* III, J1 region	SCCmec III J1 F: CATTTGTGAAACACAGTACG SCCmec III J1 R: GTTATTGAGACTCCTAAAGC	243	
	SCC*mec* II and III, *mec* complex	mecI P2: ATCAAGACTTGCATTCAGGC mecI P3: GCGGTTTCAATTCACTTGTC	209	

Table 4 – Continuation.

Assay	Target region	Primers sequence (5' to 3')	Amplicon Size (bp)	Reference
SCC*mec* typing	*mec*A gene	mecA P4: TCCAGATTACAACTTCACCAGG mecA P7: CCACTTCATATCTTGTAACG	162	(141), (122)
SCC*mec* VI subtyping	J1 region	ccrB2 F: CGAACGTAATAACATTGTCG ccrB2 R: TTGGCWATTTTACGATAGCC	203	(122), (121)
		J1IVa F: ATAAGAGATCGAACAGAAGC J1IVa R: TGAAGAAATCATGCCTATCG	278	
		J1IVb F: TTGCTCATTTCAGTCTTACC J1Ivb R: TTACTTCAGCTGCATTAAGC	336	
		J1Ivc F: CCATTGCAAATTTCTCTTCC J1Ivc R: ATAGATTCTACTGCAAGTCC	483	
		J1Ivd F: TCTCGACTGTTTGCAATAGG J1Ivd R: CAATCATCTAGTTGGATACG	575	
		J1Ivg F: TGATAGTCAAAGTATGGTGG J1Ivg R: GAATAATGCAAAGTGGAACG	792	
		J1Ivh F: TTCCTCGTTTTTTCTGAACG J1Ivh R: CAAACACTGATATTGTGTCG	663	

Table 4 – Continuation.

Assay	Target region	Primers sequence (5’ to 3’)	Amplicon Size (bp)	Reference
spa typing	*spa* gene	1113 F: TAAAGACGATCCTTCGGTGAGC 1514 R: CAGCAGTAGTGCCGTTTGCTT	variable	(2)
		spa F2: GAACAACGTAACGGCTTCATCC spa R1: CAGCAGTAGTGCCGTTTGC		(97, 140, 167)
MLST	*arcC*	arcC F2: CCTTTATTTGATTCACCAGCG arcC R1: AGGTATCTGCTTCAATCAGCG	456	(39, 55)
	aroE	aroE F1: ATCGGAAATCCTATTTCACATTC aroE R1: GGTGTTGTATTAATAACGATATC	456	
	glpF	glpF F1: CTAGGAACTGCAATCTTAATCC glpF R1: TGGTAAAATCGCATGTCCAATTC	465	
	gmk	gmk F1: ATCGTTTTATCGGGACCATC gmk R1: TCATTAACTACAACGTAATCGTA	429	
	pta	pta F1: GTTAAAATCGTATTACCTGAAGG pta R1: GACCCTTTTGTTGAAAAGCTTAA	474	
	tpi	tpi F1: TCGTTCATTCTGAACGTCGTGAA tpi R1: TTTGCACCTTCTAACAATTGTAC	402	

Table 4 – Continuation.

Assay	Target region	Primers sequence (5’ to 3’)	Amplicon Size (bp)	Reference
MLST	*yqiL*	yqiL F1: CAGCATACAGGACACCTATTGGC yqiL R1: CGTTGAGGAATCGATACTGGAAC	516	(39, 55)
ACME	*arcA*	arcA F: GAGCCAGAAGTACGCGAG arcA R: CACGTAACTTGCTAGAACGAG	671	(47)
	opp3	AIPS.45: GCAAATCTGTAAATGGTCTGTTC AIPS.46: GAAGATTGGCAGCACAAAGTG	1183	(50)
PVL	*lukS*-PV and *lukF*-PV genes	PVL 1: ATCATTAGGTAAAATGTCTGGACATGATCCA PVL 2: GCATCAASTGTATTGGATAGCAAAAGC	433	(104)

5. Questionnaire addressed to patients attending healthcare centers in Portugal, presenting skin and soft tissue infections.

Table 5 – Questions included in the questionnaire for assessment of risk factors for hospital contact addressed to patients attending healthcare centers in Portugal, presenting skin and soft tissue infections.

	Questions included in the questionnaire for risk factors assessment
1	Was hospitalized in the last 12 months?
2	Has or had been in regular contact with Geriatric Day Centers?
3	Has been infected or colonized by MRSA in the last 12 months?
4	Subjected to any surgery in the last 12 months?
5	Subjected to dialysis or permanent catheter in the last 12 months?
6.a	Has taken any antibiotic in the last 12 months?
6.b	If yes, please specify the antibiotic.

Table 6 – List of *S. aureus* isolates collected from skin and soft tissue infections of patients attending healthcare centers and respective information from the questionnaires. Legend: M - Male; F - Female; NI - no information; N - No; Y - Yes.

Strain Code	Age (years)	Sex	Sample source	Was hospitalized in the last 12 months?	Has or had been in regular contact with Geriatric Day Centers?	Has been infected or colonized by MRSA in the last 12 months?	Subjected to any surgery in the last 12 months?	Subjected to dialysis or permanent catheter in the last 12 months?	Has taken any antibiotic in the last 12 months?
CSAg1	67	M	NI	N	N	NI	N	N	Y
CSAg2	80	M	Ulcer	N	N	NI	N	N	Y
CSAg3	62	F	NI	N	N	NI	N	N	Y
CSAg4	55	M	Ulcer	N	N	N	N	N	N
CSAg5	64	M	Ulcer	N	Y	N	N	N	N
CSAg6	66	F	NI	N	N	NI	N	N	Y
CSAg9	93	F	NI	N	N	N	N	N	N
CSAg10A	68	M	NI	Y	N	N	Y	N	NI
CSAv1	37	M	Fistula	Y	N	NI	Y	N	Y
CSAv2	56	F	Ulcer	N	N	Y	N	N	Y
CSAv3	77	M	Ulcer	N	N	Y	N	N	Y
CSAv4A	68	F	Ulcer	Y	N	NI	N	N	Y
CSBa1A	93	M	Celullitis	N	Y	Y	N	N	N
CSBa4G	60	F	NI	N	N	N	N	N	Y
CSBa6	62	M	Celullitis	N	N	N	N	N	Y
CSBa8	55	F	Celullitis	N	N	N	N	N	N
CSBa10A	16	F	Celullitis	Y	N	N	Y	N	N

Table 6 – Continuation.

Strain Code	Age (years)	Sex	Sample source	Was hospitalized in the last 12 months?	Has or had been in regular contact with Geriatric Day Centers?	Has been infected or colonized by MRSA in the last 12 months?	Subjected to any surgery in the last 12 months?	Subjected to dialysis or permanent catheter in the last 12 months?	Has taken any antibiotic in the last 12 months?
CSBa12	78	M	Infected wound	N	N	N	N	N	Y
CSBa14	81	F	NI	N	N	N	N	N	Y
CSC1	77	M	Chronic wound	N	N	NI	N	N	Y
CSC2	57	M	Ulcer	N	N	NI	N	N	Y
CSC3	87	F	Ulcer	N	N	N	N	N	Y
CSC4	88	M	Ulcer	N	N	NI	NI	N	Y
CSC5	66	F	Celullitis	N	N	NI	N	N	Y
CSL1A	82	F	NI	N	N	N	N	N	Y
CSL2	77	M	Ulcer	N	N	NI	N	N	Y
CSL3	82	M	NI	N	N	NI	N	N	Y
CSL4	61	M	Ulcer	Y	Y	NI	Y	NI	Y
CSM1	57	M	NI	N	N	NI	N	N	Y
CSM2	71	F	NI	Y	N	NI	Y	Y	Y
CSM4	90	F	NI	N	Y	NI	N	N	Y
CSS1	77	M	NI	Y	N	NI	N	N	Y
CSS4	72	M	NI	N	N	NI	N	N	Y
CSS5	74	M	NI	N	N	NI	N	N	Y
CSS6	64	M	NI	Y	N	N	NI	N	Y
CSVR2	46	M	Fasciitis	N	NI	NI	N	N	Y

Table 6 – Continuation.

Strain Code	Age (years)	Sex	Sample source	Was hospitalized in the last 12 months?	Has or had been in regular contact with Geriatric Day Centers?	Has been infected or colonized by MRSA in the last 12 months?	Subjected to any surgery in the last 12 months?	Subjected to dialysis or permanent catheter in the last 12 months?	Has taken any antibiotic in the last 12 months?
CSVR3A	53	M	Fasciitis	N	N	NI	N	N	Y
CSVR4	71	M	Abcess, ulcer, fasciitis	Y	N	N	Y	N	Y
CSVR5	38	M	Abcess	N	N	Y	N	N	Y
CSVR8	81	F	Celullitis	Y	Y	NI	N	N	Y