UNIVERSIDADE NOVA DE LISBOA

'Assessment of the genetic determinants involved in the expression of high level of oxacillin resistance in contemporary clinical methicillin-resistant *Staphylococcus aureus* (MRSA) strains'

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of life threatening infections in hospital and community settings. In large part, this is due to the acquisition of resistance to ß-lactams, the most used class of antimicrobials. The majority of clinical MRSA isolates express β-lactam resistance in a heterogeneous way and several genetic factors are needed for expression of full resistance. One of the aims of our work was to identify genetic determinants responsible for the optimal expression of β-lactam resistance in strains belonging to the Brazilian clone (ST239-III). Whole genome sequencing (WGS) of two pairs of strains belonging to this clone was performed.

Our results demonstrated the presence of seven genes affected with non-synonymous mutations in both pair of strains. These genes were: *rpoB, sasC, sdrD, coa, ebh, saK, int*. Additionally we identified other genes mutated in one or the other pair of strains: *fmt, murA2, relA, valS, lysS, guaB, dfrB, gyr, tcaB, pbp2, pbp4, ccrA, blaZ, scn, cadA, mecA, merB, geh, lytA, nuc, tagB* and *pbp3*. However more studies are needed in order to confirm that the identified mutations have in fact a role in the phenotypic resistance level in the strains in study.

The mobility of the staphylococcal chromosomal cassette (SCC*mec*), which contains the *mecA* gene, the central element of methicillin resistance, is due to recombinase genes, namely *ccrA, ccrB* and *ccrC*. To assess the allelic variability of the *ccrB locus* (the most ubiquitous of the *ccr* genes) we sequenced this locus in a collection of representative MRSA strains selected in order to maximize temporal and geographical differences. The results obtained show very low mutation rate in *ccrB locus* among the clonal lineages studied. Further studies to confirm the functionality of these *ccrB* alleles and their role in the stabilization of the SCC*mec* cassettes should be performed.

Resumo

Staphylococcus aureus resistentes à meticilina (MRSA) são agentes frequentes de infeções bacterianas, a nível hospitalar e na comunidade. Uma das causas é a sua capacidade de aquisição de resistência aos β-lactâmicos, a classe de antimicrobianos mais usada em clínica. A maioria dos isolados de estafilococos expressa resistência aos β-lactâmicos de forma heterogénea e diversos fatores genéticos são necessários para a expressão total da resistência.

Um dos objetivos do trabalho apresentado nesta tese foi a identificação de determinantes genéticos responsáveis pela elevada expressão de resistência aos β-lactâmicos em estirpes do clone brasileiro (ST239-III). Com este objetivo, fizemos a sequenciação completa do genoma de dois pares de estirpes representativos deste clone.

Os resultados demonstraram a presença de sete genes afetados com mutações não-sinónimas nos dois pares de estirpes: *rpoB, sasC, sdrD, coa, ebh, sak,* e *int.* Adicionalmente, foram identificadas mutações nos genes *fmt, murA2, relA, valS, lysS, guaB, dfrB, gyr, tcaB, pbp2, pbp4, ccrA blaZ, scn, cadA, mecA, merB, geh, lyt A, nuc, tagB* e *pbp3* num ou no outro par de estirpes. Contudo, são necessários mais estudos para confirmar que as mutações encontradas e identificadas como associadas com a resistência aos β-lactâmicos têm de facto um papel no nível de resistência

A mobilização do elemento SCC*mec* que contém o gene *mecA*, o elemento central da resistência à meticilina, é devida a três genes que codificam recombinases: *ccrA*, *ccrB* e *ccrC*. De forma a estudarmos a variabilidade do gene *ccrB* (o mais comum de entre os genes *ccr*), sequenciámos este *locus* numa coleção de estirpes MRSA selecionadas, com o objectivo de maximizar diferenças tanto a nível geográfico como temporal. Os resultados obtidos sugerem um baixo grau de mutação no *locus ccrB* entre as clones estudados. Contudo serão necessários estudos complementares para confirmar o papel destes alelos *ccrB* na estabilização das cassetes SCC*mec*.

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Abbreviations

A – adenine

AA – amino acid

Arg - arginine

Asn – asparagine

Asp – aspartic acid (aspartate)

ATCC – American Type Culture Collection

bp - base pairs

C – cytosine

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

ccr – cassette chromosome recombinase

CDC – Centers for Disease Control and Prevention

CFU – colony forming units

CLSI – Clinical and Laboratory Standards Institute

DNA – deoxyribonucleic acid

dNTP – deoxynucleoside triphosphate

DR – direct repeat

EDTA – ethylenediamine tetraacetic acid

EMBL – European Molecular Biology Laboratory

ENA – European Nucleotide Archive

FOX – cefoxitin

fs – frameshift

G – guanine

gDNA – genomic deoxyribonucleic acid

Gln – glutamine

Glu – glutamic acid (glutamate)

Gly – glycine

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

His – histidine

Ile – isoleucine

IR – inverted repeat

IS – insertion sequence

Abbreviations (continuation)

kb – kilobase

kDa – kiloDalton

LTA – lipoteichoic acid

Lys – lysine

Mb – megabase

MGE – mobile genetic element

MHA – Mueller Hinton agar

MHB – Mueller Hinton broth

MIC – minimal inhibitory concentration

ml – milliliter

MLST – multilocus sequence typing

MNV – multiple nucleotide variation

MRSA – methicillin-resistant *Staphylococcus aureus*

MSSA – methicillin-susceptible *Staphylococcus aureus*

ND – not determined

ng – nanogram

NT – non typeable

OD – optical density

ORF – open reading frame

OX – oxacillin

PAP – population analysis profile

PBP – penicillin-binding protein

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PG – peptidoglycan

pmol – picomole

PVL – Panton-Valentine leukocidin

SAP – Shrimp Alkaline Phosphatase

SCC – staphylococcal cassette chromosome

Ser – serine

SNP – single nucleotide polymorphism

Abbreviations (continuation)

SNV – single nucleotide variation

Spa – *Staphylococcus aureus* protein A

ST – sequence type

T – thymine

TAE – Tris-acetate-EDTA

TBE – Tris-borate-EDTA

Thr – threonine

Tn – transposon

TSA – tryptic soy agar

TSB – tryptic soy broth

Tyr – tyrosine

V – volt

Val – valine

WGS – whole genome sequencing

WTA – wall teichoic acid

µl – microliter

Chapter I - Introduction

1. *Staphylococcus aureus* – General Features

The genus *Staphylococcus* currently has more than 40 species and several subspecies (http://www.bacterio.cict.fr/s/staphylococcus.html).

The name *Staphylococcus aureus* has its origin from the Greek word *staphylé*, that means cluster of grapes and the latin word *aureus*, that means gold (1). *S. aureus* are gram-positive cocci with approximately 1µm in diameter that divides sequentially in three orthogonal planes over three consecutive division cycles (2, 3).

S. aureus is a non-motile, non-flagellate, coagulase and catalase positive, non-spore-forming bacteria that grows in aerobic and anaerobic conditions (1).

1.1. *S. aureus* cell wall

One of the bacterial most important cellular structures is the cell wall. The cell wall is the first and major line of defense, providing the bacteria their cell shape, the strength to resist the high internal osmotic pressure and acts also as a protective barrier against threats from the environment (4). Peptidoglycan (PG), also called murein, is the main constituent of the bacterial cell wall. The PG polymer is composed by series of glycan strands of alternating N-acetylglucosamine and N-acetylmuramic acid cross-linked by short peptide bridges (5). The rigid sugar chains perpendicularly cross-linked with flexible peptide bridges results in a strong but also elastic stress-bearing structure that protects the underlying protoplast from lysing due to the high internal osmotic pressure (4, 6).

In *S. aureus* the majority of chains have a length of 3 to 10 disaccharide units. Attached to the N-acetylmuramic acid residues are stem peptides that are synthesized as pentapeptide chains, composed of L-alanyl-D-isoglutaminyl-L-lysil-D-alanyl-D-alanine. Neighbor glycan chains are cross-linked via an amino acid bridge of five glycines by the action of a transpeptidase that links the D-alanine from one stem peptide to the L-lysine of an adjacent stem peptide (6).

1.2. Colonization and infection

S. aureus is a commensal organism that colonizes the skin and mucous membranes of humans and animals (7). The anterior nares are the primary niche and the main carriage site of *S. aureus* in humans being 30% of healthy human intermittent carriers, while 20% are permanent carriers. 50% are non-carriers (8). Transmission of *S. aureus* can be accomplished by direct contact (*e.g.* via colonized hands) or indirect contact (contact with contaminated surfaces) (9). *S. aureus* is also considered an opportunistic organism because is capable of causing infection when skin and mucous membranes became disrupted (10).

S. aureus can infect almost every tissue of the human body. These infections can vary from mild skin and soft tissue infections, such as impetigo, folliculitis, cellulitis, wound and surgical infections to life-threatening infections, such as endocarditis, pneumonia, meningitis, bacteraemia and toxic shock syndrome (11).

The ability to cause severe diseases can be related to the type of strains that cause the infection and with the presence or absence of virulence factors (12).

1.3. *S. aureus* genome

S. aureus has a single circular chromosome of approximately 2,800,000 bp in size with a low DNA G+C content (average 33%) (13). The genome can be divided in core and accessory genes (14). Approximately 75% of the *S. aureus* core genome is composed by genes associated with central metabolism and housekeeping functions, such as carbohydrate metabolism, protein synthesis and DNA replication (14). Additionally to these, there are genes not essential for survival and growth, including surface binding proteins, exoenzymes, toxins, capsule proteins and virulence genes (14). The accessory genome accounts for approximately 25% of the *S. aureus* genome and encodes for nonessential functions ranging from antibiotic and metal resistance to virulence (15). The accessory genome primarily comprises mobile genetic elements (MGEs) horizontally transferred between strains such as staphylococcal cassette chromosomes (SCC), bacteriophages, genomic islands, pathogenicity islands, plasmids and transposons (14, 16). The relative ease of transfer of some MGEs between staphylococcal species, containing mainly antibiotic-resistant genes,

outstands the potential pandemic problem, not only in hospital but also in the community setting (17).

2. Beta-lactam (β-lactam) resistance in *S. aureus*

The first β-lactam antibiotic, penicillin, was discovery in 1928 by Alexander Fleming (18) but it was the work of Howard Florey and Ernest Chain with their efforts on purification and chemistry that turned penicillin into a live-saving drug (18).

The introduction of penicillin G (benzylpenicillin) into the clinical practice in the early 1940s has been recognized as one of the greatest advances in therapeutic medicine (18). Until then, the mortality rate of individuals with an *S. aureus* infection was around 80% (19).

However, soon after the introduction of penicillin, in 1942, penicillin-resistance staphylococci were observed, first in hospitals then in the community (20). These strains became resistant due to the production of β-lactamases, which are able to inactivate the penicillin. By the late 1960s, about 80% of both hospital-associated and community staphylococcal isolates were resistant to penicillin (21).

The first semi-synthetic penicillin, methicillin, originally called celbenine, was introduced into clinical practice in 1960 to avoid the β-lactamase-producing staphylococci activity (22). Methicillin has an altered β-lactam ring with two carboxyl groups (CH_3).

One year following the introduction of this antibiotic into clinical practice, the first MRSA was reported in England (23).

Although β-lactam resistance is a matter of major concern, β-lactams are still the most widely class of antibiotics prescribed, because of their high effectiveness, low cost, ease of delivery and minimal side effects (24). β-lactams in clinical use today are penicillin, narrow and extended spectrum cephalosporins, monobactams and carbapenems (25).

2.1. Mechanism of action of β-lactam antibiotics

β-lactam antibiotics are bactericidal agents that act by inhibiting the synthesis of the bacteria cell wall (26). The synthesis of PG is catalyzed by penicillin-binding proteins (PBPs), which are the bacterial targets of β-lactams. These enzymes are involved in the final stages of PG synthesis. PBPs are a group of membrane anchored extracytoplasmatic proteins that have evolved from serine proteases (27). PBPs catalyze transglycosylation and transpeptidation reactions that are required to the formation of linear glycan chains and to their crosslink, respectively (28).

β-lactams irreversibly acylate the PBP active-site serine, by mimicking the D-alanyl-D-alanine terminus of the pentapeptide side chain, by acting as substrate analogs. As consequence, the transpeptidase activity of PBPs is blocked, resulting in weakening of the cell wall and eventual lysis of the cell.

S. aureus have four native PBPs: three high-molecular-weight PBPs (PBP 1-3), and one low-molecular-weight PBP (PBP4). PBP2 is the only bi-functional PBP, with both transpeptidase and transglycosylase activities. PBP1, PBP3 and PBP4 have only transpeptidation activity.

In *S. aureus*, PBP1 and PBP2 are the minimal machinery required for PG synthesis (28). *S. aureus* also encodes other enzymes involved in PG synthesis such as MGT and SgtA, two monofunctional transglycosylases and two proteins with predicted transpeptidase activity, FmtA and FmtB (29).

2.2. β-lactam resistance in *S. aureus*

β-lactams resistance in *S. aureus* can be mediated by β-lactamase production, acquisition of an extra PBP (PBP2a) and changes in the active site of the native PBPs (30).

2.2.1. Penicillin resistance

Penicillin is inactivated by β-lactamases, encoded by *blaZ* gene, which is part of the *bla* system. β-lactamases are extracellular enzymes that promote the hydrolysis of the penicillin β-lactam ring (21). BlaR1 is a sensor/inducer transmembrane protein encoded by the *blaR1* gene and BlaI is a repressor protein, encoded by the *blaI* gene

(31). Usually, the *blaI-blaR1-blaZ* genes are carried on a plasmid or located on a transposon into the chromosome (in *S. aureus*, transposon Tn552) (32).

2.2.2. Methicillin resistance

The central element conferring resistance to methicillin and others β-lactams antibiotics is the *mecA* gene (2.1 kb in length), that encodes for PBP2a, a transpeptidase protein with low affinity for β-lactam antibiotics, being intrinsically resistant to inhibition by these antibiotics (33). In the presence of β-lactams, the synthesis of PG is assured by the cooperation between the transglycosylase activity of PBP2 and the transpeptidase activity of the acquired PBP2a (33).

The *mecA* gene, is part of a mobile genetic element found in most MRSA strains, the so-called Staphylococcal Cassette Chromosome *mec* (SCC*mec*), and is highly conserved among different lineages (34, 35). The *mecA* regulatory *locus* is a three-component system. MecR1 is a sensor/inducer protein, encoded by *mecR1* and MecR2 is an anti-repressor, encoded by *mecR2*, while MecI is a repressor protein, encoded by *mecI* gene (36).

Although the MRSA characteristic phenotype is due to *mecA* gene, about 95% of MRSA strains have the *blaZ* gene, which means that besides the SCC*mec* element, most contemporary MRSA strains still carry the β-lactamase locus (37-39). The maintenance of a functional *blaZ* gene, even in the presence of *mecA* gene could be useful as a first-line of defense against β-lactams of the first generation (penicillins), which are still widely prescribed. The fitness cost associated to the expression of a secreted enzyme (BlaZ) is likely to be smaller than the fitness cost associated to the expression of a transpeptidase protein that have to be incorporated into the bacterial cell wall (PBP2a) (40).

The transcription of *blaZ* and *mecA* are controlled by homologous regulatory systems, BlaI-BlaR1 and MecR2-MecI-MecR1, respectively (37). These systems are similar in structure, showing sequence similarity with each other and function; both exhibit corepression but not coindution (41) (see figure 1).

Each one of these regulatory systems alone is able to control the transcription of *blaZ* and *mecA* (42). The induction of *mecA* gene expression when controlled by BlaI-BlaR1 is much faster than when controlled by MecR2-MecI-MecR1, taking from few minutes to several hours, respectively. (43).

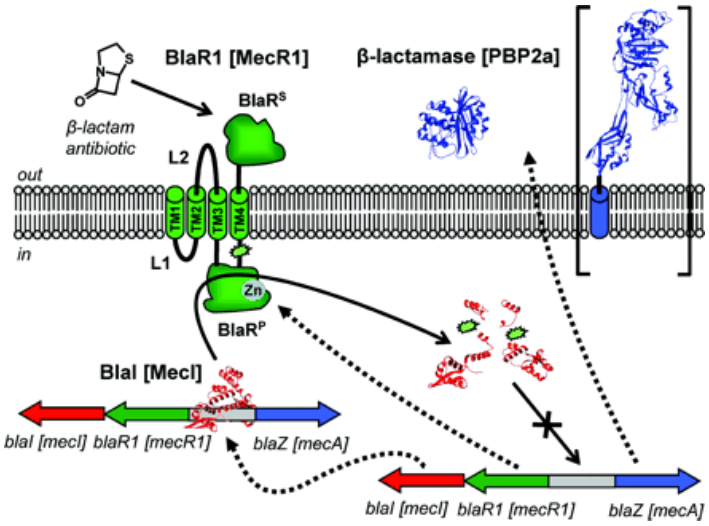


Figure 1. Regulation systems controlling the expression of β-lactamase and PBP2a (shown in large brackets) in *S. aureus*. Adapted from reference (44).

3. Staphylococcal cassette chromosome *mec* - SCC*mec*

SCC*mec* is a mobile genetic element characterized by the presence of a *mec* gene complex, containing the *mecA* gene and its regulators and by the presence of a *ccr* gene complex, containing unique site-specific recombinases named cassette chromosome recombinases (Ccr) and finally by flanking direct and inverted repeats (45, 46).

When a MSSA exogenously acquires the SCC*mec*, by horizontal transfer, a MRSA is generated (47, 48).

The *mec* gene complex is characterized by the *mecA* gene, intact or truncated copies of the *mecA* regulatory genes, namely *mecI*, *mecR1* and *mecR2* and a copy of the insertion sequence IS431.

In *S. aureus* there are three major *mec* classes. Class A has intact sequences for *mecI-mecR1* while classes B and C do not have *mecI* and have a partially deleted *mecR1* (46).

The *ccr* gene complex is composed by the *ccr* genes and surrounding open reading frames (ORFs). Cassette chromosome recombinases (Ccr) are large serine recombinases of the resolvase/invertase family. Their main function is the excision and integration of the SCC*mec* element, *i.e.* the SCC*mec* mobilization.

Ccr recombinases are encoded by three phylogenetically different *ccr* genes, *ccrA*, *ccrB* and *ccrC*, with nucleotide correspondences among them below 50%. Usually, *ccr* genes with nucleotide identities more than 85% are assigned to the same allotype (42). To date, *ccrA* is classified into five allotypes, *ccrB* into seven allotypes and *ccrC* in two (49).

The gene *ccrB* is the most ubiquitous in SCC*mec* in MRSA and displays more sequence diversity than *ccrA* and *mecA* (50).

According to the guidelines proposed by the International Working Group on the Classification of SCC elements, SCC*mec* types are defined by the type of *ccr* gene complex and the class of the *mec* gene complex. Besides these two main components, SCC*mec* has also three regions called J, for joining (J1-3). These three hypervariable regions (J1-J2-J3), constitute nonessential components of the SCC*mec*, and include antibiotic resistance genes, pseudogenes, transposons, plasmids and insertion sequences. SCC*mec* subtypes are defined by the J regions. A SCC*mec* is considered non-typeable (NT) when it is not possible to ascertain a class of *mec* complex and/or a type of *ccr* (57).

The structural organization of SCC*mec* may be summarized as J1-ccr-J2-mec-J3 (46).

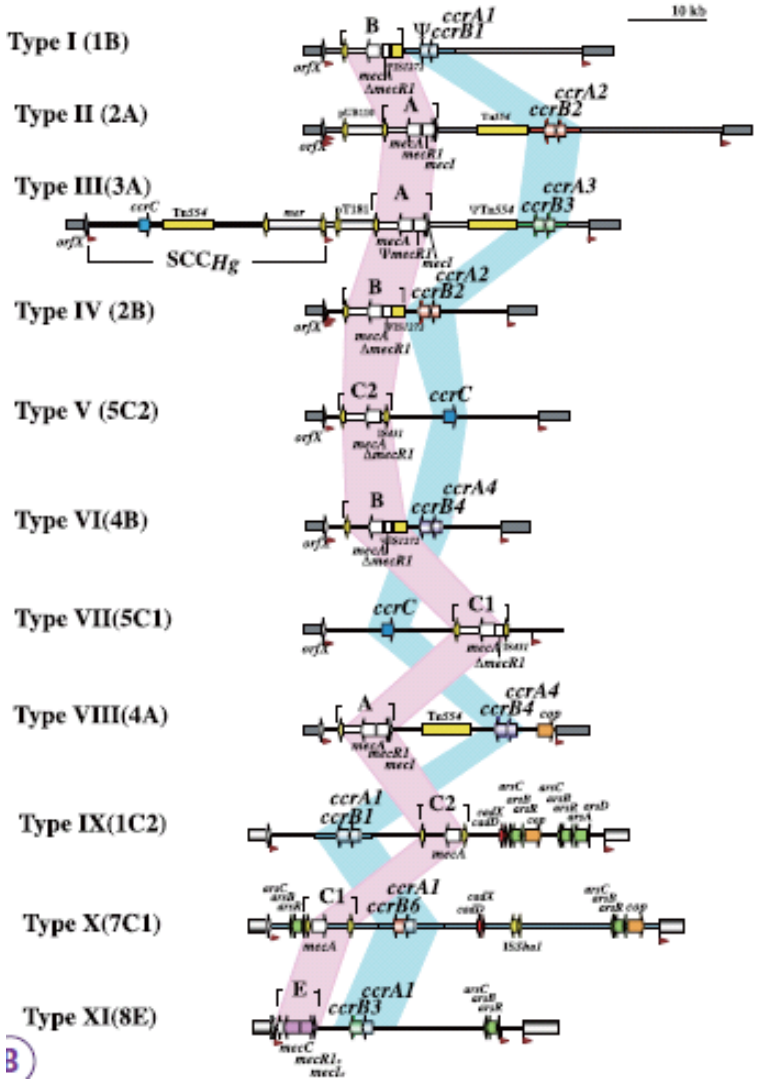


Figure 2. Types of SCC*mec* (I-XI) identified in *S. aureus*. Adapted from reference(55).

To date, twelve different SCC*mec* types have been described (49) (see Table 1 and Figure 2).

Table 1. SCC*mec* types identified in *S. aureus*. Adapted from http://www.sccmec.org.

SCC*mec* type	*ccr* gene complex	*ccr* gene complex structure (allotypes)	*mec* gene complex	*mec* gene complex structure
I	Type 1	A1B1	B	IS431-*mecA*-Δ*mecR1*-IS1272
II	Type 2	A2B2	A	IS431-*mecA*-*mecR1*-*mecI*
III	Type 3	A3B3	A	IS431-*mecA*-*mecR1*-*mecI*
IV	Type 2	A2B2	B	IS431-*mecA*-Δ*mecR1*-IS1272
V	Type 5	C1	C2	IS431-*mecA*-Δ*mecR1*-IS431(the two IS431s are arranged in the opposite direction)
VI	Type 4	A4B4	B	IS431-*mecA*-Δ*mecR1*-IS1272
VII	Type 5	C1	C1	IS431-*mecA*-Δ*mecR1*-IS431(the two IS431s are arranged in the same direction)
VIII	Type 4	A4B4	A	IS431-*mecA*-*mecR1*-*mecI*
IX	Type 1	A1B1	C2	IS431-*mecA*-Δ*mecR1*-IS431(the two IS431s are arranged in the opposite direction)
X	Type 7	A1B6	C1	IS431-*mecA*-Δ*mecR1*-IS431(the two IS431s are arranged in the same direction)
XI	Type 8	A1B3	E	*blaZ*-*mecA*$_{LGA251}$-*mecR1*$_{LGA251}$-*mecI*$_{LGA251}$
XII	Type 9	C2	C2	IS431-*mecA*-Δ*mecR1*-IS431(the two IS431s are arranged in the opposite direction)

SCC*mec* types range in size from 21 to 67 kb and SCC*mec* III is considered to be the longest SCC*mec* element with 67 kb in length and it consists of two SCC elements, SCC*mec* type III and SCCmercury, harboring *ccrC*, integrated plasmid pl258 and tranposon Tn554. (46)

3.1. Integration and excision of SCC*mec* elements

SCC*mec* elements are large pieces of DNA that are integrated at the 3´end of an open reading frame (*orfX*) in the *S. aureus* chromosome, at a specific site referred as *attB* (Figure 3), located near the replication origin, which is 66 kb – 89 kb upstream of the *spa* gene and 10 kb downstream of *purA*, depending on the size of the integrated SCC*mec* (51-53).

OrfX is a staphylococcal ribosomal methyltransferase of the RlmH type. OrfX methylates 70S ribosomes and is conserved in all staphylococci (54).

After integration, SCC*mec* is flanked by direct repeats (DR) sequences, which serve as integration sites for the next SCC element (55).

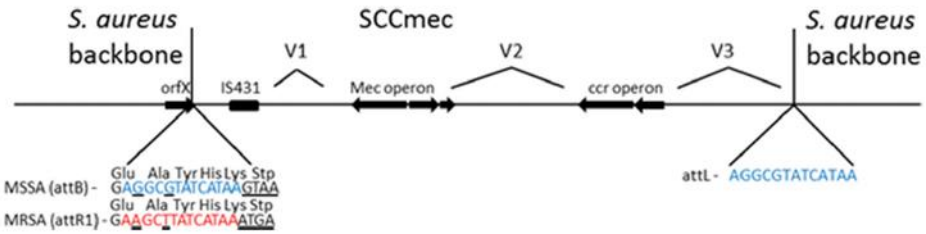


Figure 3. **Schematic illustration of the general arrangement of SCC*mec*.** Adapted from reference (54).

3.2. Origin and evolution of the SCC*mec*

The origin of SCC*mec* is still unclear. It was suggested that *Staphylococcus sciuri* harbored the ancestor of the gene *mecA* encoding PBP2a. Most *S. sciuri* are methicilin susceptible but in the presence of methicillin these bacteria could become resistant to the antibiotic due to an increase in the transcription of the *mecA* homologue caused by the appearance of mutations in the *mecA* promoter region (56). It has been proposed that the MRSA had been originated in vivo through the horizontal transfer of *mecA* gene from coagulase negative staphylococci (57).

3.3. MRSA clones

MRSA clones are defined by the combination of their multilocus sequence type (MLST) and by the SCC*mec* type they carry (*e.g.* ST239-SCC*mec* III, abbreviated as ST239-III) (58).

The first MRSA strain (NCTC10442) was isolated in 1961, in the United Kingdom and harbored SCC*mec* type I. This MRSA clone is known as the Archaic clone and was disseminated all over the world in the 1970s and 1980s. In 1982, in Japan, the

MRSA strain (N315) that harbored SCC*mec* type II was isolated. Strains with the characteristics of N315, were assigned to the New York/Japan clone that spread worldwide in the 1990s. Only three years later, in 1985, in New Zealand, another MRSA strain (85/2082) harboring SCC*mec* type III was isolated (48, 59). In the 1990s, some MRSA clones harboring SCC*mec* type IV have spread all over the world. In 2004, it was isolated in Australia, the MRSA strain WIS that harbored SCC*mec* type V. Other MRSA clones have been identified as described below (45, 60-64).

Some of the major MRSA clones included the Brazilian (ST239-III), New York/Japan (ST5-II), EMRSA 15 (ST22-IV_h), EMRSA 16 (ST36-II), Berlin (ST45-II), Pediatric (ST5-VI and ST5-IV_a), USA 300 (ST8-IV_a), European (ST80-IV_{NT} and ST80-IV_c), Southwest/Pacific (ST30-IV) clone (65) (Table 2 and 3). The Brazilian clone (ST239) is one of the most successful and persistent clones, is multiantibiotic-resistant and accounts for 90% of hospital-associated MRSA (HA-MRSA) around the world. This clone spread throughout Asia, South America and is actually in Eastern Europe (66).

The appearance and spread of different methicillin-resistant clones over time has been referred to as a wave-like phenomenon.

The first wave is associated with penicillin-resistant *S. aureus*, the second wave stands in for methicillin-resistant *S. aureus*, the third wave representing the hospital-associated MRSA (HA-MRSA) and the fourth wave is related to community-associated MRSA (CA-MRSA) in the late 1990s (67). Since then, CA-MRSA has emerged worldwide, not only in the community but also in healthcare facilities. Nowadays, the difference between HA-MRSA and CA-MRSA is beginning to fade (31, 68), it has been reported the presence of major HA-MRSA clones in the community and CA-MRSA clones as being the cause of nosocomial outbreaks.

CA-MRSA strains (*e.g* strains usually carrying SCC*mec* type IV and type VI) when compared with HA-MRSA strains (*e.g.* strains usually carrying SCC*mec* type I-III) tend to be more virulent due to the presence of various virulence factors, faster growth, frequently susceptible to non-β-lactams classes of antibiotics and to have a lower degree of β-lactam resistance (69). The smaller size of some CA-MRSA strains such as type SCC*mec* type IV allow the rapid dissemination of MRSA clones with these SCC*mec* types, usually by horizontal gene transfer, presumably via bacteriophages (70).

Table 2. An overview of the major HA-MRSA clones. Adapted from reference (**65**).

HA-MRSA clone	MLST profile[a]	ST[b]	CC[c]	SCC*mec*[d]
Archaic	3-3-1-1-4-4-16	250	8	I
Berlin (USA600)	10-14-8-6-10-3-2	45	45	IV
Brazilian/Hungarian	2-3-1-1-4-4-3	239	8	III
Iberian	3-3-1-12-4-4-16	247	8	I
Irish-1	3-3-1-1-4-4-3	8	8	II
New York/Japan (USA100)	1-4-1-4-12-1-10	5	5	II
Pediatric (USA800)	1-4-1-4-12-1-10	5	5	IV
Southern Germany	1-4-1-4-12-24-29	228	5	I
UK EMRSA-2/-6 (USA500)	3-3-1-1-4-4-3	8	8	IV
UK EMRSA-3	1-4-1-4-12-1-10	5	5	I
UK EMRSA-15	7-6-1-5-8-8-6	22	22	IV
UK EMRSA-16 (USA200)	2-2-2-2-3-3-2	36	36	II

[a] Multilocus sequence typing.
[b] Sequence type.
[c] Clonal complex.
[d] Staphylococcal cassette chromosome *mec*.

Table 3. An overview of the major PVL-positive CA-MRSA clones. Adapted from reference (**65**).

CA-MRSA clone	MLST profile[a]	ST[b]	CC[c]	SCC*mec*[d]
European	1-3-1-14-11-51-10	80	80	IV
Southwest Pacific (USA1100)	2-2-2-2-6-3-2	30	30	IV
USA400	1-1-1-1-1-1-1	1	1	IV
USA300[g]	3-3-1-1-4-4-3	8	8	IV
USA1000	19-23-15-2-19-20-15	59	59	IV or VII

[a] Multilocus sequence typing.
[b] Sequence type.
[c] Clonal complex.
[d] Staphylococcal cassette chromosome *mec*.

4. Heterogeneous β-lactam resistance

Several studies demonstrated that MRSA strains, highly resistant to β-lactam antibiotics and beta-lactamase producers, can have a nonfunctional MecI-MecR1 regulatory system. This inactivation can occur either through deletion of the *mecI* gene or by accumulation of point mutations within the *mecI* coding sequence or in the *mecA* promoter with subsequent increase in the production of PBP2a. Nevertheless, there are some cases reported in which strains phenotypically resistant to methicillin have no mutations in the *mecI* nor in the *mecA* promoter region (72). The amount of PBP2a does not fully correlate with the resistance level (73).

It has been reported the existence of genetic determinants that are responsible for the high β-lactam resistance levels in subpopulations of MRSA (72, 74). Mutations in genes that encode for products associated with distinct functional categories, such as transcription, translation, transport, cell division among others, have been associated with highly resistant MRSA strains; some examples are, *rpoB, murE*, *murF*, *femB, femX*, *hmrA*, *glmM*, *fmtA*, *dacA*, *sigB*, *spoVG*, *sarA*, *xdrA*, *ccpA*, *secDF*, *vraR*, *vraS*, *agrA*, *agrB* (75).

Most contemporary MRSA strains present heterogeneous methicillin resistance. Such hetero-resistant MRSA strains have the majority of the cells resistant to relatively low or moderate concentrations of antibiotic, sometimes near the minimal inhibitory concentration (MIC) threshold of a susceptible strain, while subpopulations are able to grow at a much higher antibiotic MIC value (76). As a result, clinical isolates of MRSA can be improperly diagnosed and the use of β-lactams might lead to treatment failure (77).

5. Whole genome sequencing (WGS)

The first *S. aureus* genomes were sequenced in 2001 (51) and in the past fifteen years, the costs for performing a WGS analysis have drastically decrease, allowing this technique to became, in the near future, part of the routine typing methods (78).

Several sequencing technologies are been used, from “sequencing by ligation” to “sequencing by synthesis” approaches. As each sequencing technology has strengths

and weaknesses, the choice of a specific platform to perform the WGS have to be selected in a way to obtain the desired experimental results.

One of the platforms commonly used is MySeq (Illumina Inc., San Diego, CA, USA), in which "sequencing by synthesis" consists in individual DNA molecules being attached to the surface of flow cells and isothermal ´bridging` amplification is used to amplify signals that are sequenced by using reversible fluorophore-labeled nucleotides, which are optically read from each flow cell. These techniques have low error rates, produce large amounts of raw data and have high accuracy, which provides sufficient discrimination between isolates of the same MRSA clone. One of the disadvantages is that individual read lengths tend to be short and this can be a problem for genomes with large repeats (78).

The applications of genome sequencing go from Clinical medicine, Bacterial evolution, Genomic archaeology to Metagenomics studies. Currently, full genome sequences of a large number of MRSA strains are available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

The power of genome sequencing to identify multidrug-resistant pathogens is an invaluable tool to infection control (79).

Point mutations are the most frequent sequence variations in bacterial genomes. These point mutations result in single nucleotide polymorphisms (SNPs). There are SNP differences in housekeeping and other stable genes. Isolates belonging to the same lineage have a remarkably conserved core genome, even when separated by time and space (80).

The workflow of bacterial sequencing consists in sample preparation, library preparation, DNA sequencing, sequence assembly (*de novo* or against a reference) and bioinformatics analysis (78).

6. Aim

This thesis is composed by two different studies (study A and study B).

Study A was performed in order to identify the genetic determinants that could be on the basis of the different level of β-lactam resistance in strains belonging to the same genetic background and characterized by the same SCC*mec* type. Thus, two pairs of strains, belonging to the Brazilian clone, with the same genetic background (as ascertain by their MLST sequence type and/or *spa* type) and SCC*mec* types but with different β-lactam resistance profiles were selected. The two pairs of strains were compared by whole genome sequencing and the presence of non-synonymous mutations that could explain the phenotypic differences in the β-lactam resistance between the strains selected was analyzed.

Study B was developed to assess the allelic variability of the *ccrB locus* in successful clonal lineages for which the SCC*mec* stability remains to be determined (EMRSA15, EMRSA16, Berlin, Pediatric, USA300, European, SW/Pacific and Brazilian clones). Functionality assays will be performed in the future using the different alleles identified in this study in order to infer the role of the different *ccrB* alleles in the stabilization of the SCC*mec* elements in these particular clones.

Chapter II – Materials and Methods

Study A: Assessment of the genetic determinants involved in the expression of high level of beta-lactam resistance in contemporary clinical MRSA strains

1. Strain collection

The methicillin-resistant *Staphylococcus aureus* (MRSA) strains used in the present study are listed in Supplementary Table 1S (Annex 1). Seventy-five MRSA strains previously characterized as belonging to the Brazilian clone, with multilocus sequence type 239 (ST239), a *spa* type usually associated with this clone, and carrying SCC*mec* type III were selected from the collection of the Laboratory of Molecular Genetics, Microbiology of Human Pathogens Unit from Instituto de Tecnologia Química e Biológica, Oeiras, Portugal.

2. Media and growth conditions

S. aureus strains were grown overnight at 37°C on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) or on Mueller Hinton agar (MHA, Becton Dickinson, Sparks, MD, USA) or tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) or Mueller Hinton broth (MHB, Becton Dickinson, Sparks, MD, USA) under aerobic conditions.

3. Phenotypic analysis of β-lactam resistance in *S. aureus*

The susceptibilities of *S. aureus* strains to oxacillin and cefoxitin were determined by disk diffusion method, Etest (bioMérieux SA, Marcy-l`Étoile, France) and population analysis profiles (PAPs).

3.1. Disk diffusion method

The disk diffusion method (Kirby-Bauer) was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (81).

Briefly, a small aliquot of overnight cultures (grown in TSB at 37ºC) was diluted in MHB to an optical density at 620nm (OD_{620}) of 0.08 (equivalent to 0.5 McFarland turbidity) were spread on MHA plates, followed by placing oxacillin (OX) diffusion disks with 1 µg of antibiotic (Oxoid, Basingstoke, UK) and cefoxitin (FOX) diffusion disks with 30 µg of antibiotic (Oxoid, Basingstoke, UK) on the plates.

Minimum inhibitory concentration (MIC) values were evaluated after 18h incubation at 35ºC.

S. aureus American Type Culture Collection (ATCC) strain 25923, a *mecA* negative isolate, was used as a quality control strain for the disk diffusion method. Results were only considered when the oxacillin and the cefoxitin disk halos for ATCC 25923 were 18-24 mm and 23-29 mm, respectively (81).

The following CLSI breakpoints for testing the susceptibility of *S. aureus* were used: oxacillin MIC susceptible (S) ≥ 13 mm, intermediate (I) = 11-12 mm, resistant (R) ≤ 10 mm; cefoxitin MIC susceptible (S) ≥ 22 mm and resistant (R) ≤ 21 mm.

3.2. Etest

Etest was performed according to the CLSI guidelines. Briefly, a small aliquot of overnight cultures (grown in TSB at 37ºC) was diluted in MHB to an optical density at 620nm (OD_{620}) of 0.08 (equivalent to 0.5 McFarland turbidity) were spread on Mueller Hinton agar plates (MHA, Becton Dickinson, Sparks, MD, USA), followed by placing the oxacillin (OX) or cefoxitin (FOX) Etest strips (bioMérieux SA, Marcy-l`Étoile, France) on MHA plates supplemented with 2% NaCl at 35°C for 24h.

The following CLSI breakpoints for defining the susceptibility of *S. aureus* were used: oxacillin MIC susceptible (S) ≤ 2 µg/ml and resistant (R) ≥ 4 µg/ml; cefoxitin MIC susceptible (S) ≤ 4 µg/ml and resistant (R) ≥ 8 µg/ml.

3.3. Population analysis profiles (PAPs)

Population analysis profiles were performed for two pairs of strains (CPS68 vs HDG2 and HU107 vs HGSA145), by the agar plate method (76), in order to confirm their MICs.

Briefly, 25 µl of 10^{0}, 10^{-1}, 10^{-3}, 10^{-5} dilutions of an overnight culture (grown in TSB at 37°C) were plated on TSA plates containing increasing concentrations of cefoxitin (Sigma-Aldrich). Colony forming units (CFU) were counted for every dilution with non-confluent growth after 48 h of incubation at 37°C.

4. DNA isolation

Chromosomal DNA from the two pairs of strains (CPS68 vs HDG2 and HU107 vs HGSA145) was prepared by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer`s recommendations, except for the lysis step with lysostaphin at 0.5 mg/ml and RNase at 0.3 mg/ml.

5. SCC*mec* typing

For the identification of the structural types/subtypes of the staphylococcal chromosomal cassette (SCC*mec*) and in order to confirm that the pairs of strains selected carried the same type/subtype of SCC*mec*, the Multiplex polymerase chain reaction (PCR) strategy described by Oliveira *et al.* in 2002 was performed as previously described (82).

The following prototype strains were used: COL as reference for SCC*mec* type I, N315 for SCC*mec* type II, ANS46 for SCC*mec* type III, HU25 for SCC*mec* IIIA, HDG2 for SCC*mec* IIIB and MW2 for SCC*mec* type IV.

PCR amplifications were performed in a T1 Thermocycler (Biometra, Alfagene). The PCR products (10 µl) were resolved in a 2% Seakem LE Agarose (Lonza, Rockland, ME USA), using the ladder 1-kb DNA Ladder Plus (Thermo Scientific, Fermentas) (Annex 2) in 0.5X Tris-borate-EDTA buffer (TBE) at 100 V for 45 min. Gels were

stained with ethidium bromide (0.15 µg/ml), then visualized and recorded under UV light in GelDoc XR$^+$ System (Bio-Rad, Hercules, California, USA).

Uniplex PCR reactions were performed in dubious cases, with the same primers used in the multiplex methodology. Detection of *mecA*, *mecI*, RIF4, RIF5, IS431, pT181 and *dcs* was done in a final volume of 25 µl; containing 5 ng of the DNA template, 1X GoTaq Flexi buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$ (Promega, Madison, WI, USA), 160 µM of each dNTP (Bioron), 10 pmol of each primer, 0.625U of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA).

The cycling conditions were: predenaturation at 94°C for 4 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 45 s; a final extension at 72°C for 4 min and soaking at 16°C. These assays were performed in a T1 Thermocycler (Biometra, Alfagene).

The PCR products (10 µl) were detected by separation in a 1% Seakem LE Agarose (Lonza, Rockland, ME USA) using the ladder 1-kb DNA Ladder Plus (Thermo Scientific, Fermentas) in 1X Tris-acetate-EDTA buffer (TAE), at 80 V, for 40 min. Gels were stained with ethidium bromide (0.15µg/ml), then visualized and recorded under UV light in GelDoc XR$^+$ System (Bio-Rad, Hercules, California, USA).

6. Whole genome sequencing (WGS)

6.1. DNA isolation

Genomic DNA (gDNA) from strains HU107, HGSA145 and CPS68 was extracted using an optimization of the Qiagen DNeasy Blood & Tissue Kit (Qiagen) protocol (for details see Annex 3).

Samples were considered pure when the absorbance (A260/A280) ratio was between 1.7 and 1.9 and absorbance scans showed a symmetric peak at 260 nm. The DNA concentration was determined using the Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington, USA) and the Qubit 2.0 Fluorometer (Invitrogen, life technologies, Carlsbad, CA, USA).

6.2. Genome sequencing

Whole genome sequencing of MRSA strains CPS68, HU107 and HGSA145 was performed using the Illumina MiSeq platform at Instituto Gulbenkian de Ciência, Oeiras, Portugal, with a minimum coverage of 30X.

The coverage of the sequenced samples was confirmed using the following formula:

$$\text{Coverage} = \frac{(\text{number of reads after trim}) \times (\text{avg. length after trim})}{3{,}0\ \text{Mb}}$$

6.2.1. Detection of variations

Paired-end reads from each strain were mapped using CLC Genomics Workbench 8.0.1 (Qiagen Aarthus, Denmark) against the chromosome of the reference *Staphylococcus aureus* ST239 strain TW20 (16) to identify single nucleotide variants (SNVs), multi nucleotide variants (MNVs), deletions, insertions and replacements in the genome. WGS data from strain HDG2 were obtained from the European Nucleotide Archive (ENA) database (www.ebi.ac.uk/ena/data/view/ERS049891).

The sequence and annotation of the TW20 genome were obtained from EMBL database with accession number FN433596 (16).

Variants analysis was performed with the tool "Quality-based variant detection", using CLC Genomics Workbench 8.0.1.

Study B: Assessment of allelic variation in the *ccrB* locus in MRSA clones

7. Strain collection

MRSA strains used in this study are listed in Table 4. Eighty MRSA strains belonging to the EMRSA15 (ST22-IVh), EMRSA16 (ST36-II), Berlin (ST45-IV and ST45-V), Pediatric (ST5-VI), USA300 (ST8-IVa), European (ST80-IV_{NT} and ST80-IVc), Southwest Pacific (ST30-IV) and Brazilian (ST239-III) clonal lineages and previously characterized in terms of their genetic background by *spa* typing and/or MLST and in terms of their SCC*mec* type, were selected from the MRSA culture collection of the Laboratory of Molecular Genetics at Instituto de Tecnologia Química e Biológica, Oeiras, Portugal. The selection of the strains was done with the objective to maximize temporal and geographical differences.

Table 4. Strains used in study B.

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type
EMRSA15	DEN4561	2001	Denmark	t022	22	IV
(ST22-IVh)	HU303	2003	Hungary	t032	22	IV
n = 10	C331	2009	Hungary	t020	22	IV_h
	HDE461	NA	Portugal	t022	ND	IV
	HGSA158	2003	Portugal	t849	22	IV_h
	HDES57	2007	Portugal	t032	22	IV_h
	HDES102	2008	Portugal	t032	ND	IV_h
	ICBAS12	2009	Portugal	t032	ND	IV_h
	ICBAS46	2010	Portugal	t032	ND	IV_h
	C124	2009	UK	t032	22	IV_h
EMRSA16	HU275	2002	Hungary	t018	36	II
(ST36-II)	HPH2	2006	Portugal	t018	ND	II
n = 4	C10	1998	USA	t018	36	II
	SJ-101	2013	USA	t018	36	II

Table 4. Strains used in study B. (continuation).

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type
Berlin (ST45-IV) (ST45-V) n = 7	1150/93	NA	NA	t004	45	ND
	DEN3050	2001	Denmark	t015	45	IV
	HU281	2002	Hungary	t038	45	IV
	PLN49	1997	Poland	t015	45	IV
	D17	1998	USA	ND	45	IV
	SJ-511	2013	USA	t214	ND	IVa
	HDES79	2007	Portugal	t004	45	IVa
Pediatric (ST5-IVa) n = 12	ARG164	1994-1996	Argentina	t002	ND	IV
	CLB1	1996	Colombia	t002	ND	IV
	DEN698	2001	Denmark	t002	5	IV
	HPV17	1992-1993	Portugal	t311	5	VI
	HDE65	1993-1994	Portugal	t311	ND	VI
	HDE1	1992	Portugal	t311	5	VI
	HDE383	1997	Portugal	t311	ND	VI
	HDES26	2007	Portugal	t062	5	VI
	HDES93	2007	Portugal	t002	ND	IVc
	ICBAS43	2010	Portugal	t002	5	IV_a
	C380	2005	Spain	t311	5	IV_a
	STP46A	2012	STP	t105	5	IV_a
USA300 (ST8-IVa) n=11	CR1	1996	CZ	t024	8	IV
	CR43	1996	CZ	t008	8	IV
	DEN2988	2001	Denmark	t008	8	IVa
	HU288	2002	Hungary	t008	8	IV
	HU394	2005	Hungary	t008	8	IVa
	IPOP65	2001	Portugal	t024	8	ND
	ICBAS10	2009	Portugal	t008	8	IVa
	C368	2004	Spain	t121	8	IVa
	C377	2005	Spain	t008	8	IVa
	STP151	2012	STP	t064	8	IV_{NT}
	USA300	1995-2003	USA	ND	8	IVa

Table 4. Strains used in study B. (continuation).

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type
European	C006	2002	CZ	t044	80	IV_c
(ST80-IV_{NT})	C014	2002	CZ	t131	80	IV_c
(ST80-IV_c)	DEN18851	1995	Denmark	t044	80	IV (multiplex v1)
n=13	DEN4250	1996	Denmark	t044	80	IV (multiplex v1)
	DEN11819	1997	Denmark	t044	80	IV (multiplex v1)
	DEN2948	2001	Denmark	t376	80	IV
	E31	1997	Finland	t044	80	IV (multiplex v1)
	HT0401	2002	France	t044	80	IV (multiplex v1)
	C206	2005	Greece	t044	80	IV_{NT}
	HU376	2002	Hungary	t044	80	IV_c
	HFF189	2005	Portugal	t044	80	IV
	C273	2005	Romania	t067	80	IV_c
	02-1418	2002	The Netherlands	t044	80	IV (multiplex v1)
SW/Pacific	C017	2004	CZ	t019	30	IV_c
(ST30-IV)	SJ-031	2012	USA	t021	30	IVa
n = 4	SJ123	2013	USA	t019	30	IVc
	DEN45	2001	Denmark	t018	30	NT (multiplex v1)
Brazilian	AGT120	1997	Argentina	t037	ND	ND
	BZ48	1997	Brazil	t037	239	IIIA
(ST239-III)	CHL1	1997	Chile	t037	ND	ND
n = 19	CHL151	1998	Chile	t037	ND	ND
	GRE18	1998	Greece	t037	239	III
	GRE317	1999	Greece	t138	239	IIIA
	HU125	1994	Hungary	t037	239	III
	HUR4	1997	Hungary	t787	239	III
	HU248	2001	Hungary	t037	239	III
	HU270	2002	Hungary	t030	239	III
	HU272	2002	Hungary	t787	239	III
	HU294	2003	Hungary	t538	239	IIIA
	HGSA15	1994	Portugal	t037	239	IIIA
	HGSA57	1995	Portugal	t037	239	IIIA
	HGSA142	2003	Portugal	t037	239	IIIA
	TAW97	1998	Taiwan	t037	239	IIIA
	TUR4	1996	Turkey	t030	239	III
	URU34	1997	Uruguay	t037	ND	ND
	URU110	1998	Uruguay	t037	ND	ND

ST, sequence type; ID, identification; ND, not determined; NA, not available; NT, non typeable; SW, Southwest; var, variant UK, United Kingdom; USA, United States of America; CZ, Czech Republic; STP, São Tomé and Príncipe.
Note: the *ccrB* allele from strains highlighted in grey were extrapolated from WGS data.

8. Media and growth conditions

Strains were grown overnight at 37°C on tryptic soy agar (TSA, Becton Dickinson, Sparks, MD, USA) or tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA) under aerobic conditions.

9. DNA isolation

Chromosomal DNA was extracted using the boiling method (see Annex 4 for details) and in dubious cases, DNA was prepared by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer`s recommendations, except for the lysis step with lysostaphin at 0.5 mg/ml and RNase at 0.3 mg/ml.

10. *ccrB* sequencing

Seventy-one out of the 80 MRSA strains in study were analyzed using the sequencing approach described by Oliveira *et al.* in 2006 (83), in which the allelic variation in the *ccrB locus* is evaluated by sequencing internal fragments of *ccrB* amplified by PCR, using a pair of degenerated primers (ccrB F1 and ccrB R1). In each reaction (final volume of 50 µl), 5 ng of the DNA template, 1X PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems), 160 µM of each dNTP (Bioron), 100 pmol of each primer, and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems) was used. PCR amplifications were performed in a T1 Thermocycler (Biometra, Alfagene) with the following cycling conditions: predenaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, 42°C for 60 s, 72°C for 2 min; a final extension at 72°C for 4 min and soaking at 16°C.

The PCR products (5 µl) were detected by separation in a 1% Seakem LE Agarose (Lonza, Rockland, ME USA) using the ladder 1-kb DNA Ladder Plus (Thermo Scientific, Fermentas) in 1X Tris-acetate-EDTA buffer (TAE), at 80 V, for 40 min. Gels were stained with ethidium bromide (0.15 µg/ml) then visualized and recorded under UV light in GelDoc XR^+ System (Bio-Rad, Hercules, California, USA).

After the visualization of the PCR products, the amplified fragments were purified using a mix of Exonuclease I and Shrimp Alkaline Phosphatase (SAP) enzymes, as described in the protocol provided by Dag Harmsen, Ridom Bioinformatics.
Briefly, to 30 µl of amplified PCR product, 6 µl of Exonuclease I (1U/µl) (New England Biolabs) and 6 µl of SAP (1U/µl) (USB Amersham) were added. The mixture was then subjected to a cycling program on the PCR machine with the following conditions: 30 min at 37°C followed by 20 min at 80°C.
The *ccrB* allele of nine strains was extrapolated from the available WGS data (strains highlighted in grey in Table 4), obtained from the European Nucleotide Archive (ENA) database (www.ebi.ac.uk/ena.).

11. *ccrB* allelic variation

DNA sequencing of both strands (forward and reverse) were performed at Macrogen (Amsterdam, The Netherlands)

The *ccrB* trace sequences were analyzed with DNA Star software (Lasergene, Madison, WI, USA) and alleles were assigned in accordance with the previously attributed *ccrB* alleles deposited in the *ccrB* database of the Laboratory of Molecular Genetics at Instituto de Tecnologia Química e Biológica, Oeiras, Portugal.

Chapter III – Results

Study A: Assessment of the genetic determinants involved in the expression of high level of beta-lactam resistance in contemporary clinical MRSA strains

In order to analyze the genetic determinants responsible for the optimal expression of β-lactam resistance in strains belonging to the Brazilian clone, whole genome sequencing (WGS) for two pairs of strains belonging to the Brazilian clone (ST239-III) sharing identical genetic backgrounds but with different cefoxitin resistance profiles was performed.

1. Strain selection

Antimicrobial susceptibility tests to oxacillin and cefoxitin were performed in order to select two pairs of strains characterized by the same ST and/or *spa* types but with dissimilar oxacilin and/or cefoxitin resistance profiles (Supplementary Table 1S – in annexes section).

A subset of strains (HGSA339/HGSA145 and HU42/HU103) sharing identical genetic backgrounds and with discrepant values in the oxacillin disk halos were selected in order to determine their oxacillin MIC values by Etest.

Table 5. Oxacillin resistance profiles of a subset of strains in study.

Strain	Isolation year	Isolation origin	SCC*mec* type	SCC*mec* subtype	*spa* type	Sequence type (MLST)	Oxacillin resistance	
							Disk diffusion (mm)	Etest (µg/ml)
HGSA339	2003	Portugal	III	IIIA	t037	ND	6	256
HGSA145	2003	Portugal	III	IIIA	t037	ND	9	256
HU42	1995	Hungary	III	ND	t787	ND	6	2
HU103	1996	Hungary	III	ND	t787	ND	15	8

Since no correlation could be established between the values of the oxacillin disk halos and the oxacillin MIC values determined by Etest (Table 5), oxacillin resistance profiles were replaced in favor of cefoxitin resistance profiles. Cefoxitin is used as a substitute for *mecA*-mediated oxacillin resistance according to CLSI guidelines (84).

When compared to oxacillin, cefoxitin is a better inducer of the *mecA* gene and tests using cefoxitin give more accurate and reproducible results than tests with oxacillin (www.cdc.gov).

A correlation between the values of cefoxitin disk halos and MICs determined by Etest could be established for a new subset of strains (HU107/HGSA145 and CPS68/HDG2) sharing identical genetic backgrounds but with discrepant values in the cefoxitin disk halos (Table 6).

Table 6. Cefoxitin resistance profiles of a subset of strains in study.

Strain	Isolation year	Isolation origin	SCC*mec* type	SCC*mec* subtype	*spa* type	Sequence type (MLST)	Cefoxitin resistance	
							Disk diffusion (mm)	Etest (µg/ml)
HU107	1996	Hungary	III	ND	t037	ND	12	48
HGSA145	2003	Portugal	III	IIIA	t037	ND	6	256
CPS68	1985	Portugal	III	ND	t421	239	13	48
HDG2	1993	Portugal	III	IIIB	t421	239	6	256

Two pairs of strains were selected for further study: Pair A composed by HU107 and HGSA145, both characterized by *spa* type t037 and with cefoxitin MICs of 48 and 256 µg/ml, respectively (Figure 4); and Pair B composed by CPS68 and HDG2, both characterized by *spa* type t421 and with cefoxitin MICs of 48 and 256 µg/ml, respectively (Figure 5).

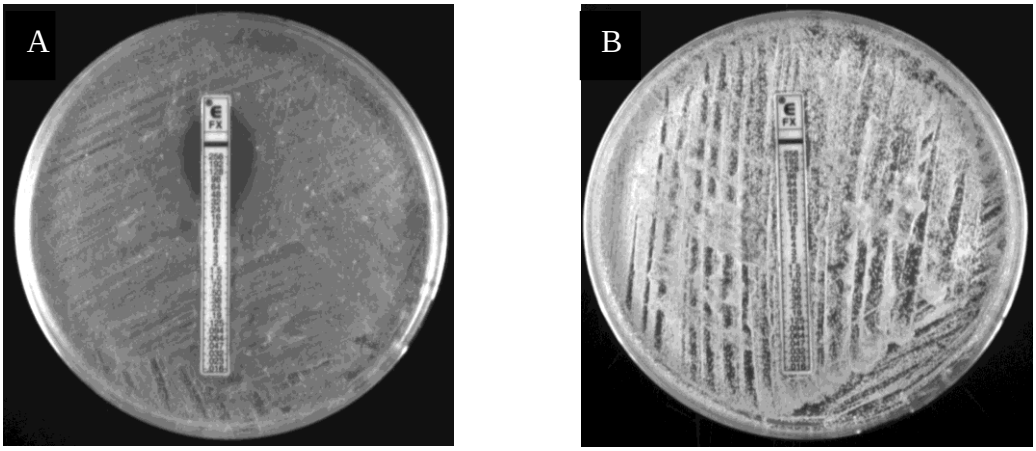


Figure 4. Cefoxitin susceptibility determined by Etest for strains HU107 and HGSA145. A. HU107, MIC = 48 µg/ml and B. HGSA145, MIC = 256 µg/ml.

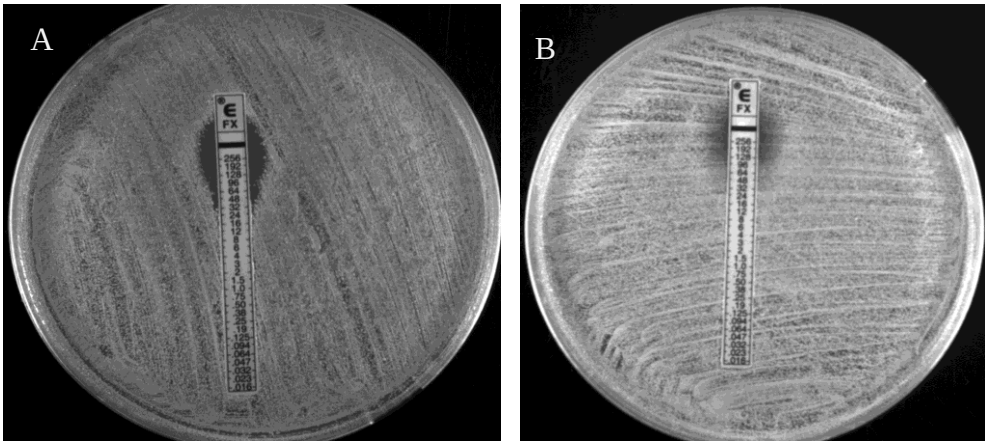


Figure 5. Cefoxitin susceptibility determined by Etest for strains CPS68 and HDG2. A. CPS68, MIC = 48 µg/ml and B. HDG2, MIC = 256 µg/ml.

2. Population analysis profiles (PAPs) results

Population analysis profiles were performed for both pairs of strains in order to confirm their MICs and the differences in the MIC values of the strains belonging to the same pair. (Figures 6 and 7).

Pair A composed by strains HU107 and HGSA145, exhibited an MIC value of 12.5 µg/ml and 200 µg/ml as determined by PAP, respectively (Figure 6).

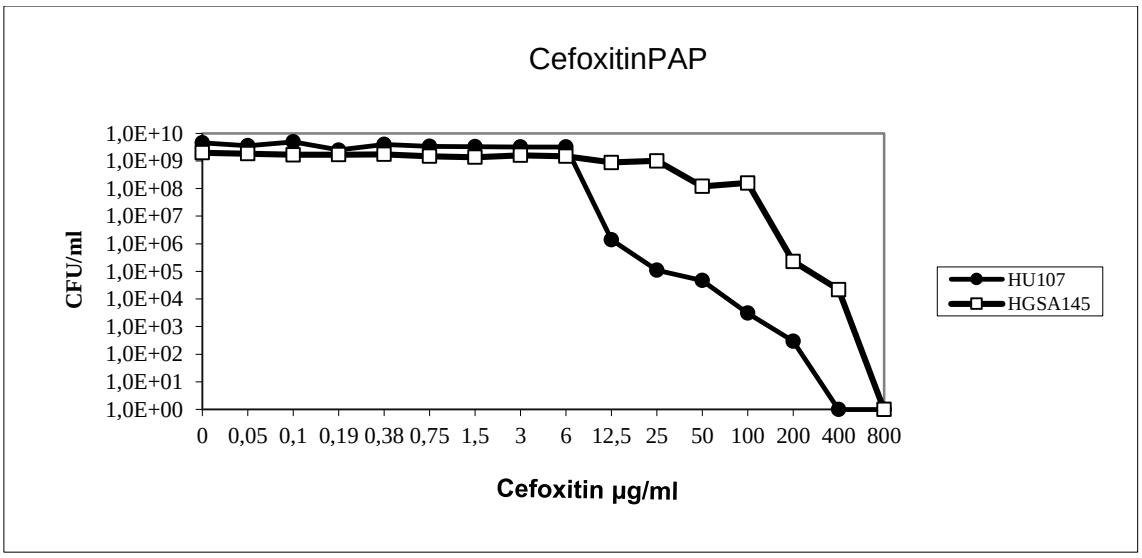


Figure 6. Cefoxitin susceptibility determined by population analysis profile for strains HU107 and HGSA145. A. HU107, MIC = 12.5 µg/ml and B. HGSA145, MIC = 200 µg/ml. Colony-forming units (CFU) were determined by counting colonies after 48h of incubation on TSA plates at 37°C.

Pair B composed by strains CPS68 and HDG2, showed an MIC value of 25 µg/ml for both strains as determined by PAP using TSA plates and 48h of incubation at 37°C (Figure 7).

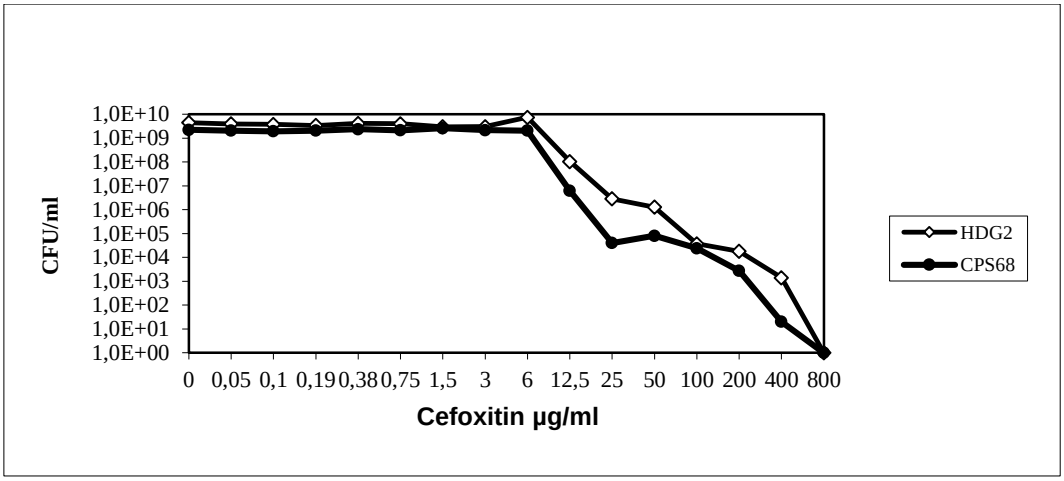


Figure 7. Cefoxitin susceptibily determined by population analysis profile for strains CPS68 and HDG2. A. CPS68, MIC = 25 µg/ml and B. HDG2, MIC = 25 µg/ml. Colony-forming units (CFU) were determined by counting colonies after 48h of incubation on TSA plates at 37°C.

Taking into account that the Etest results were significantly different from the results obtained with the PAPs, it was decided to determine the cefoxitin resistance for this pair of strains in different growth conditions, namely at a different temperature - 30°C instead of 37°C.

When the growth temperature of the PAPs was altered from 37°C to 30°C, using TSA as growth media, the MIC results were similar to the ones obtained by Etest, with CPS68 and HDG2, exhibiting MIC values of 50 and 200 µg/ml, respectively (Figure 8).

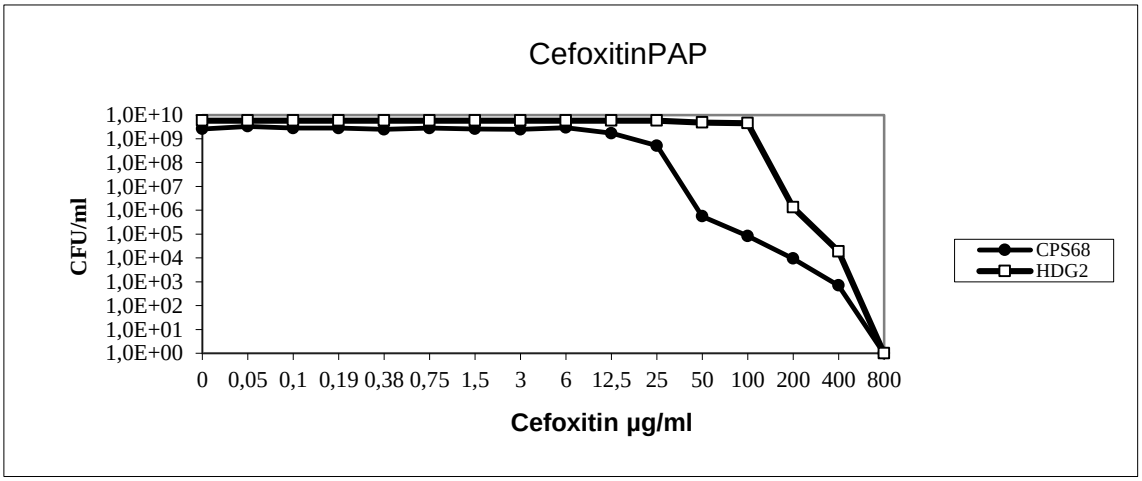


Figure 8. Cefoxitin susceptibility determined by population analysis profile for strains CPS68 and HDG2. A. CPS68, MIC = 50 µg/ml and B. HDG2, MIC = 200 µg/ml. Colony-forming units (CFU) were determined by counting colonies after 48h of incubation on TSA plates at 30°C.

3. SCC*mec* results

In order to confirm that the pair of strains selected carried the same SCC*mec*, SCC*mec* typing was performed using the methodology described by Oliveira *et al.* in 2002 (82).

Strains COL, N315, ANS46 and MW2 were included as controls for SCC*mec* type I, II, III and IV, respectively; and strains HU25 and HDG2 were used as controls for subtypes IIIA and IIIB, respectively. Since, some of the patters were not clearly defined due to weak bands in the agarose gel, uniplex PCR reactions were additionally performed to confirm the dubious results.

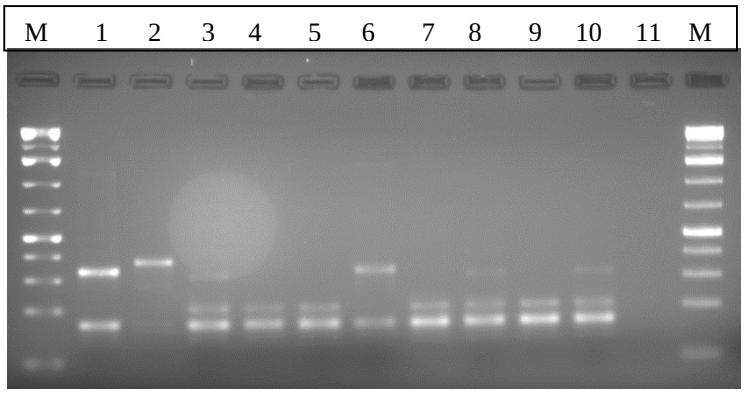


Figure 9. Multiplex PCR results for SCC*mec* types identification. Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – COL (SCC*mec* type I); lane 2 – N315 (SCC*mec* type II); lane 3 – ANS46 (SCC*mec* type III); lane 4 – HU25 (SCC*mec* type IIIA); lane 5 – HDG2 (SCC*mec* type IIIB); lane 6 – MW2 (SCC*mec* type IV); lane 7 – HGSA145; lane 8 – HU107; lane 9 -HDG2; lane 10 – CPS68; lane 11 – negative control

Uniplex PCR reactions were performed for the detection of *mecA*, *mecI*, RIF4, RIF5, IS431, pT181 (Figures 10-14).

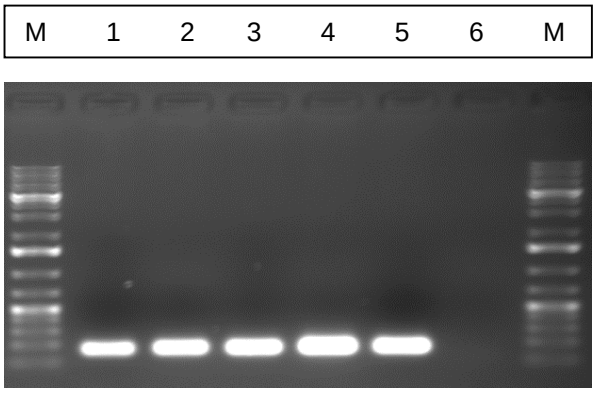


Figure 10. PCR results for the detection of *mecA* (expected amplified fragment of 162 bp). Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 - ANS46 (positive control); lane 2 - HGSA145; lane 3 – HU107; lane 4 – HDG2; lane 5 – CPS68; lane 6 – negative control.

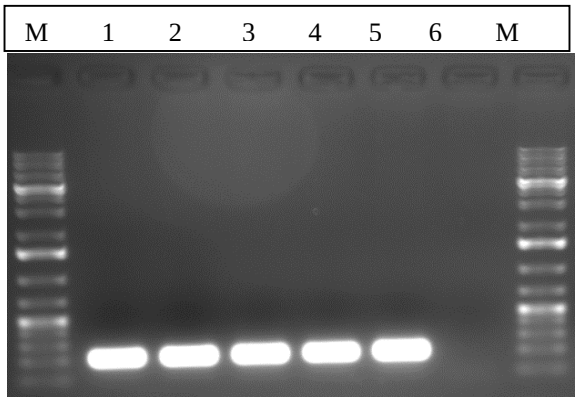


Figure 11. PCR results for the detection of *mecI* (expected amplified fragment of 243 bp). Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – ANS46 (positive control); lane 2 – HGSA145; lane 3 - HU107; lane 4 – HDG2; lane 5 – CPS68; lane 6 – negative control.

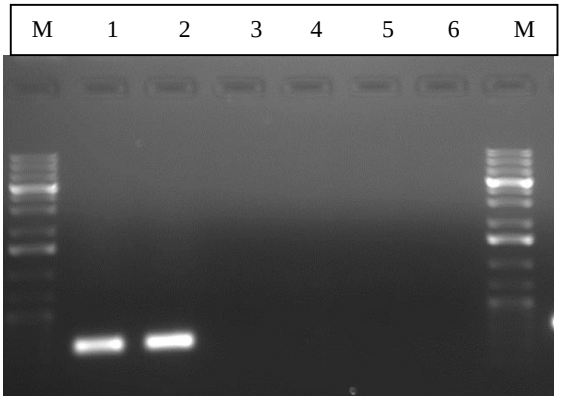


Figure 12. PCR results for the detection of RIF4 (expected amplified fragment of 243 bp). Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – ANS46 (positive control); lane 2 – HGSA145; lane 3 - HU107; lane 4 – HDG2; lane 5 – CPS68; lane 6 – negative control.

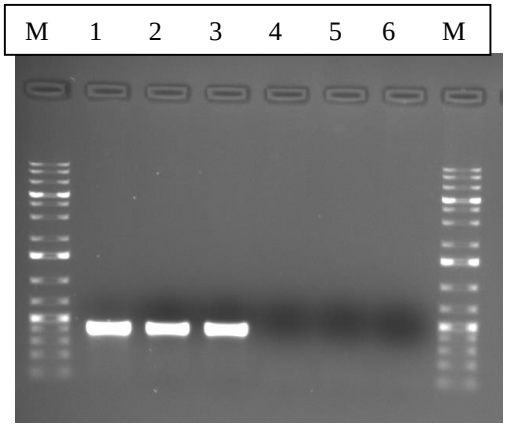


Figure 13. PCR results for the detection of RIF5 (expected amplified fragment of 414 bp).
Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – ANS46 (positive control); lane 2 – HGSA145; lane 3 - HU107; lane 4 – HDG2; lane 5 – CPS68; lane 6 – negative control.

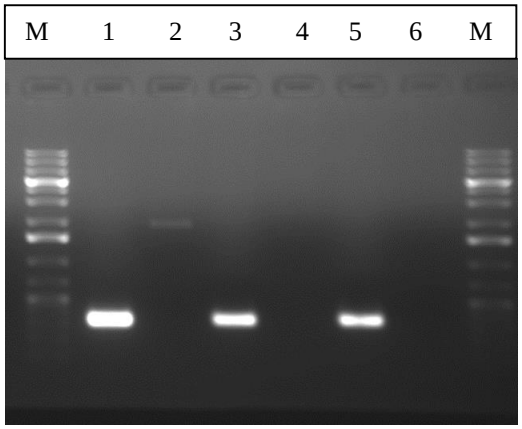


Figure 14. PCR results for the detection of pT181 and IS431 (expected amplified fragment of 303 bp). Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – ANS46 (positive control); lane 2 – HGSA145; lane 3 - HU107; lane 4 – HDG2; lane 5 – CPS68; lane 6 – negative control.

The combined results obtained in the SCC*mec* multiplex PCR and in the uniplex PCRs confirmed that the two pairs of strains selected were composed by strains belonging to the same SCC*mec* type (type III), but with different subtypes. Pair A is constituted by strains carrying SCC*mec* types III (HU107) and IIIA (HGSA145) and Pair B is pair B constituted by strains carrying SCC*mec* types III (CPS68) and IIIB (HDG2). Since the

differences between the SCC*mec* III subtypes reside in the presence or absence of antibiotic resistance determinants for non-β-lactam antibiotics it was decided to pursue with the WGS analysis with the selected pair of strains.

4. Preparation of genomic DNAs for WGS

To verify the chromosomal DNA integrity of the strains HGSA145, HU107 and CPS68 after the gDNA extraction using an optimization of the Qiagen DNeasy Blood & Tissue Kit (Qiagen) protocol, 2 µl of the extraction products were detected by separation in a 1% Seakem LE Agarose (Lonza, Rockland, ME USA) using the 1 kb DNA Ladder Plus (Thermo Scientific, Fermentas) in 1 X Tris-acetate-EDTA buffer (TAE), at 100 V, for 1h. Gels were stained with ethidium bromide (0.15 µg/ml), then visualized and recorded under UV light in GelDoc XR$^+$ System (Bio-Rad, Hercules, California, USA) (Figure 13).

With the gel electrophoresis results we could observe that the chromosomal DNAs were intact and do not present any state of degradation, since the bands were well defined and without smear.

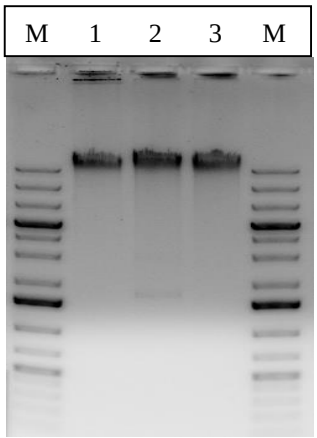


Figure 15. Visualization of the genomic integrity. Lanes are as follows: lane M – 1 kb DNA Ladder Plus; lane 1 – HGSA145; lane 2 – HU107; lane 3 – CPS68

The DNA concentration was determined using the Nanodrop 1000 spectrophotometer (Thermo scientific, Wilmington, USA) and the Qubit 2.0 Fluorometer (Invitrogen, life technologies, Carlsbad, CA, USA). The results obtained with Nanodrop and Qubit

respectively were as follow - HU107: 128 ng/µl and 192 ng/µl; HGSA145: 88.6 ng/µl and 154 ng/µl and for CPS68: 53.3 ng/µl and 65.6 ng/µl respectively.

5. WGS results

SNVs, MNVs, deletions, insertions and replacements detection

In order to identify differences at the genomic level between the two pairs of strains selected previously (HU107/HGSA145 and CPS68/HDG2), WGS was performed.
The WGS samples coverage was confirmed to be 33X for HU107, 32X for HGSA145, 51X for CPS68 and 91X for HDG2. After the sequences trimming (the trimming percentage was around 99.9% for each strain and the number of reads after trim were for pair A HU107/HGSA145 376,291 and 331,989 respectively and for pair B CPS68/HDG2 594,861 and 3,653,828 respectively) (for details see Trim summary in annexes section, annex 5-8), pair-end reads were mapped to the reference *Staphylococcus aureus* ST239 strain TW20 genome. In a first step the strains from both pairs were compared with the reference strain *Staphylococcus aureus* TW20. Next, all the mutations shared between strains of the same pair, i. e., mutations of the same type in the same nucleotide position, were filtered out, making possible the comparison of mutations between the two strains of each pair (HU107 against HGSA145 and CPS68 against HDG2). Only non-synonymous mutations were considered and these mutations were divided in core and accessory regions.

SNVs at sites with heterogeneous mappings were filtered out if the SNV was present in less than 75% of the reads at that site.
The mutated gene, their product and the nature of the nucleotide and amino acid change are listed in Tables 2-7 deposited in the following link: http://www.itqb.unl.pt/~m.gabrielabento/.
The number of non-synonymous mutations, in strains of pair A (HU107 vs HGSA145) demonstrated that SNVs (point mutations) are the most frequent mutation in accessory, and core regions (Table 7). This was also true for strains belonging to pair B (CPS68/HDG2) (Table 8).

Table 7. Number of non-synonymous mutations in pair A (HU107/HGSA145)

Genomic Region	Type of mutations	Number of non-synonymous mutations
Accessory (n=298)	SNV	239
	MNV	42
	deletion	7
	insertion	10
	replacement	0
Core (n=295)	SNV	269
	MNV	8
	deletion	12
	insertion	6
	replacement	0

Table 8. Number of non-synonymous mutations in pair B (CPS68/HDG2).

Genomic Region	Type of mutations	Number of non-synonymous mutations
Accessory (n=295)	SNV	195
	MNV	71
	deletion	8
	insertion	12
	replacement	9
Core (n=88)	SNV	80
	MNV	3
	deletion	4
	insertion	1
	replacement	0

The genomic regions with the higher number of mutations were different in the two pairs of strains: in pair A the two regions (accessory and the core) have similar number of mutations, whereas in pair B the accessory region has a higher number of mutations.

Large part of the non-synonymous mutations in the accessory genome of both pair of strains are affecting genes coding phage proteins and genes belonging to pathogenic genomic islands (http://www.itqb.unl.pt/~m.gabrielabento/). Among these, there are mutations in genes related with virulence (*sak* in pair A; *sak* and *scn* in pair B), resistance to antibiotics and heavy metals (*merB* and *mecA* in pair A and *cadA* and *blaZ* in pair B), in genes coding for proteins with recombinase and integrase functions (*ccrB*, *ccrC* and *int* in pair A and *ccrA* and *int* in pair B), and in genes coding for proteins involved in phage life cycle (*geh* and *lytA* in pair A).

Regarding the mutations affecting the core genome in pair A (http://www.itqb.unl.pt/~m.gabrielabento/) there were non-synonymous mutations in genes related with resistance to antibiotics (*rpoB*, *fmt*, *murA2*, *relA*, *valS*, *lysS*, *guaB*, *dfrB*, *gyrA*, *grlA*, *tcaB*, *pbp2*, *pbp4* and genes coding for Mur ligase family of proteins in pair A; *rpoB* in pair B), in genes related with virulence (*coa*, *nuc*, *isdl*, in pair A; *coa* in pair B), genes coding for proteins with integrase function (*int* in both pairs), genes coding for surface-anchored *S. aureus* binding proteins that interact with human matrix molecules (*sdrD*, *sasC* and *ebh* in both pairs), genes coding for proteins related with cell wall (*pbp3* and *tagB* in pair A).

The comparison of genes with non-synonymous mutations in the two pairs of strains revealed the presence of genes sharing the same nucleotide change that gives rise to the same amino acid change. Table 9 lists the genes affected with non-synonymous mutations that are common to the two pairs of strains.

Table 9. Non-synonymous mutations shared by the two pairs of strains in study.

Gene (locus in *S. aureus* TW20)	Description	Nucleotide change in Pair A[a] (HU107→HGSA145)[a]	Amino acid change in pair A	Nucleotide change in pair B[a] (CPS68→HDG2)[a]	Amino acid change in pair B
rpoB (SATW20_06120)	DNA-directed RNA polymerase beta chain protein	C657724A	His481Asn	C657696A C657724A	Asp471Glu His481Asn
sasC (SATW20_17460)	LPXTG surface protein	T1901860C	Asn1670Asp	T1903840C	Lys1010Glu
sdrD (SATW20_06320)	Serine-aspartate repeat-containing protein D	A687539C	Lys843Gln	C686889T	Pro626Leu
coa (SATW20_02310)	Staphylocoagulase precursor	G268880A C269376A G269379C A269416C G269422-AC269428CA	Gly390Asp Tyr555* Lys556Asn Asn569His Asp571Fs Thr573Gln	C269254A -269260G T269293C C269295A CA269266AC	Asn515His Asp517Fs Gln528* Gln528His Thr519Gln
ebh (SATW20_14350)	Very large surface anchored protein	A1539129G C1539288T C1551654A A1557405G C1558485T	Gln7534* Lys7481Glu Ala3359Ser His1442Tyr Gly1082Ser	A1552133G	Thr3199Met
Sak (SATW20_19380)	Staphylokinase precursor	C2111512T	Val159Ile	C2111512T C2111778T G2111862A T2111974C	Val159Ile Arg70His Ala42Val Ser5Gly
Int (SATW20_20000)	integrase	T2152593C T2152774G	Ile206Thr His266Gln	C2152025T A2152646G G2152752C	His17Tyr Asn224Asp Gly259Ala

[a]C657724A, C at position 657724 changed to A; G269422-, G at position 269422 was deleted; -269260G, insertion of a G at position 269260. *Stop codon. Fs, Frameshift.
Note: Highlighted in grey are mutations of the same kind shared by the two pairs of strains.

Study B: Assessment of allelic variation in the *ccrB* locus in MRSA clones

The evaluation of the allelic variation in *ccrB* genes among MRSA strains belonging to the same genetic lineages was determined using the sequencing approach described by Oliveira *et al.* in 2006 (83). The *ccrB* variability was evaluated for the successful clonal lineages for which the SCC*mec* stability remains to be determined, *i.e.* for Brazilian (ST239-III), EMRSA15 (ST22-$IV_{h)}$, EMRSA16 (ST36-II), Berlin (ST45-IV), Pediatric (ST5-$IV_{a)}$, USA300 (ST8-IV_a), European (ST80-IV), SW/Pacific (ST30-IV) clones.

6. *ccrB* allelic variation results

Seven different alleles were identified in the eight clones selected for this study (Table 10).

Table 10. *ccrB* allelic variation found in strains from study B.

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type	*ccrB* allele
EMRSA15	DEN4561	2001	Denmark	t022	22	IV	400
(ST22-IVh)	HU303	2003	Hungary	t032	22	IV	400
n = 10	C331	2009	Hungary	t020	22	IV_h	400
	HDE461	NA	Portugal	t022	ND	IV	400
	HGSA158	2003	Portugal	t849	22	IV_h	400
	HDES57	2007	Portugal	t032	22	IV_h	400
	HDES102	2008	Portugal	t032	ND	IV_h	400
	ICBAS12	2009	Portugal	t032	ND	IV_h	400
	ICBAS46	2010	Portugal	t032	ND	IV_h	400
	C124	2009	UK	t032	22	IV_h	400
EMRSA16	HU275	2002	Hungary	t018	36	II	200
(ST36-II)	HPH2	2006	Portugal	t018	ND	II	200
n = 4	C10	1998	USA	t018	36	II	200
	SJ-101	2013	USA	t018	36	II	200
Berlin	1150/93	NA	NA	t004	45	ND	400
(ST45-IV)	DEN3050	2001	Denmark	t015	45	IV	400
(ST45-V)	HU281	2002	Hungary	t038	45	IV	401
n = 7	PLN49	1997	Poland	t015	45	IV	400
	D17	1998	USA	ND	45	IV	400
	SJ-511	2013	USA	t214	ND	IVa	400
	HDES79	2007	Portugal	t004	45	IVa	400
Pediatric	ARG164	1994-1996	Argentina	t002	ND	IV	400
(ST5-IVa)	CLB1	1996	Colombia	t002	ND	IV	400
n = 12	DEN698	2001	Denmark	t002	5	IV	400
	HPV17	1992-1993	Portugal	t311	5	VI	600
	HDE65	1993-1994	Portugal	t311	ND	VI	600
	HDE1	1992	Portugal	t311	5	VI	400
	HDE383	1997	Portugal	t311	ND	VI	600
	HDES26	2007	Portugal	t062	5	VI	600
	HDES93	2007	Portugal	t002	ND	IVc	400
	ICBAS43	2010	Portugal	t002	5	IV_a	401
	C380	2005	Spain	t311	5	IV_a	400
	STP46A	2012	STP	t105	5	IV_a	401

Table 10. *ccrB* allelic variation found in strains from study B (continuation).

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type	*ccrB* allele
USA300	CR1	1996	CZ	t024	8	IV	409
(ST8-IVa)	CR43	1996	CZ	t008	8	IV	400
n=11	DEN2988	2001	Denmark	t008	8	IVa	400
	HU288	2002	Hungary	t008	8	IV	412
	HU394	2005	Hungary	t008	8	IVa	400
	IPOP65	2001	Portugal	t024	8	ND	400
	ICBAS10	2009	Portugal	t008	8	IVa	400
	C368	2004	Spain	t121	8	IVa	400
	C377	2005	Spain	t008	8	IVa	400
	STP151	2012	STP	t064	8	IV_{NT}	400
	USA300	1995-2003	USA	ND	8	IVa	400
European	C006	2002	CZ	t044	80	IV_c	400
($ST80\text{-}IV_{NT}$)	C014	2002	CZ	t131	80	IV_c	400
($ST80\text{-}IV_c$)	DEN18851	1995	Denmark	t044	80	IV (multiplex v1)	400
n=13	DEN4250	1996	Denmark	t044	80	IV (multiplex v1)	400
	DEN11819	1997	Denmark	t044	80	IV (multiplex v1)	400
	DEN2948	2001	Denmark	t376	80	IV	400
	E31	1997	Finland	t044	80	IV (multiplex v1)	400
	HT0401	2002	France	t044	80	IV (multiplex v1)	400
	C206	2005	Greece	t044	80	IV_{NT}	400
	HU376	2002	Hungary	t044	80	IV_c	400
	HFF189	2005	Portugal	t044	80	IV	400
	C273	2005	Romania	t067	80	IV_c	400
	02-1418	2002	The Netherlands	t044	80	IV (multiplex v1)	400
SW/Pacific	C017	2004	CZ	t019	30	IV_c	400
(ST30-IV)	SJ-031	2012	USA	t021	30	IVa	401
n = 4	SJ123	2013	USA	t019	30	IVc	400
	DEN45	2001	Denmark	t018	30	NT (multiplex v1)	400

Table 10. *ccrB* allelic variation found in strains from study B (continuation).

Clone	Strain ID	Isolation year	Isolation origin	*spa* type	MLST	SCC*mec* type	*ccrB* allele
Brazilian	AGT120	1997	Argentina	t037	ND	ND	300
	BZ48	1997	Brazil	t037	239	IIIA	300
(ST239-III)	CHL1	1997	Chile	t037	ND	ND	300
n = 19	CHL151	1998	Chile	t037	ND	ND	300
	GRE18	1998	Greece	t037	239	III	300
	GRE317	1999	Greece	t138	239	IIIA	300
	HU125	1994	Hungary	t037	239	III	300
	HUR4	1997	Hungary	t787	239	III	300
	HU248	2001	Hungary	t037	239	III	new allele
	HU270	2002	Hungary	t030	239	III	300
	HU272	2002	Hungary	t787	239	III	300
	HU294	2003	Hungary	t538	239	IIIA	300
	HGSA15	1994	Portugal	t037	239	IIIA	300
	HGSA57	1995	Portugal	t037	239	IIIA	300
	HGSA142	2003	Portugal	t037	239	IIIA	300
	TAW97	1998	Taiwan	t037	239	IIIA	300
	TUR4	1996	Turkey	t030	239	III	300
	URU34	1997	Uruguay	t037	ND	ND	300
	URU110	1998	Uruguay	t037	ND	ND	300

ST, sequence type; ID, identification; ND, not determined; NA, not available; NT, non typeable; SW, Southwest; var, variant; UK, United Kingdom; USA, United States of America; CZ, Czech Republic; STP, São Tomé and Príncipe.
Note: the *ccrB* allele from strains highlighted in grey were extrapolated from WGS data.

6.1. *ccrB* allelic variation among strains belonging to the same MRSA clone

All strains belonging to EMRSA15 (SCC*mec* $IV_{h)}$ are characterized by the presence of *ccrB* allele 400, strains from EMRSA16 (SCC*mec* II) presented a *ccrB* allele 200, strains belonging to European clone (SCC*mec* $IV_{c \text{ and } NT}$) presented an *ccrB* allele 400. The remaining clonal lineages (Berlin, Pediatric, USA300, SW/Pacific and Brazilian) were characterized by the presence of more than one *ccrB* allele.

A new allele was identified in the strain HU248 that belongs to the Brazilian clone.

Chapter IV – Discussion and conclusions

The adaptation power of methicillin-resistant *Staphylococcus aureus* (MRSA) to the antibiotic environment makes these bacteria one of the most dangerous pathogens worldwide. MRSA has been considered over the past few decades one of the most important hospital-associated human pathogens and has also recently emerged as a community-associated pathogen. The acquisition of methicillin resistance in *S. aureus* is associated with the expression of an extra penicillin-binding protein (PBP2a), encoded by the *mecA* gene and conferring cross-resistance to virtually all β-lactams antibiotics. Although the *mecA* gene is essential, other genetic factors besides the methicillin genetic determinant are needed for the optimal expression of β-lactam resistance in *S. aureus*. The knowledge of the genetic determinants involved in the β-lactam resistance mechanism in MRSA is essential for the proper design of new antimicrobials useful to combat infections caused by this serious and pandemic pathogen. It was initially believed that the worldwide spread of MRSA was solely due to the vertical transfer of methicillin resistance through clonal expansion of successful clones; however, it is now estimated that the *de novo* acquisition of SCC*mec* elements carrying the methicillin resistance genetic determinant by horizontal transfer occurs at a high frequency, contributing for its rapid dissemination among *S. aureus*. Despite this estimation, few predominant clonal lineages dominate the structure of contemporary MRSA. Factors contributing for the predominance of particular clones and for the maintenance of their SCC*mec* elements are not clear. Mutations in the recombinases responsible for the mobility of these SCC*mec* elements may lead to the loss of the recombinase function and consequently to the stabilization of the SCC*mec* element in a particular clonal lineage. One of the most relevant MRSA clones responsible for major epidemic outbreaks worldwide is the multiresistant Brazilian clone that carries determinants of resistance to antibiotic and heavy metals and also virulence factors.

The work presented in this thesis was developed in order to identify genetic determinants involved in the establishment of high levels of β-lactam resistance in some MRSA strains belonging to the Brazilian clonal lineage and to assess the allelic variability among the *ccrB* gene in several predominant MRSA clonal lineages.

Study A: Assessment of the genetic determinants involved in the expression of high level of beta-lactam resistance in contemporary clinical MRSA strains

In order to identify the genetic determinants involved in the expression of high-level resistance to β-lactams in contemporary clinical MRSA strains, two pairs of strains belonging to the Brazilian clone and sharing the same genetic background, as assessed by multi-locus sequence type (ST), *spa* type, and SCC*mec* cassette, but with different levels of cefoxitin resistance were selected.

Chromosomal DNA of strains HU107 and HGSA145 (pair A) and strains CPS68 and HDG2 (pair B) was analyzed by whole genome sequencing (WGS) in order to identify mutations in genes that could explain the different levels of cefoxitin resistance between them. Therefore, only non-synonymous mutations were considered in the study.

In order to simplify the whole genome sequencing analysis, the genome data were divided in accessory and core regions.

As expected, the accessory genome presented a high number of mutations in both pair of strains. Point mutations were the most frequent variation found in accessory and core regions (Tables 7 and 8).

The accessory genome plays an important role in bacterial survival, allowing rapid adaptation via horizontal gene transfer (85-87).

The presence of mobile genetic elements such as bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands and transposons are common in MRSA strains (14). In this study and for both pairs of strains, phage proteins and pathogenicity islands accounted for the major part of the products with alteration. In order to identify genetic determinants that may be involved in the establishment of high levels of cefoxitin resistance in some strains belonging to the Brazilian clone, the affected genes in both pair of strains studied were compared. There were seven affected genes in common in the two pairs of strains, two from the accessory genome and five from the core genome. In the accessory genome, the mutations were detected in the genes *sak* that encodes for staphylokinase and is contained in a prophage (88) and in the gene *int*, which encodes for an integrase protein. Both pairs of strains, presented the same nucleotidic change at the nucleotidic position 2111512 in the *sak*

gene: a change from a cytosine (found in the most susceptible isolates – HU107 and CPS68) to a thymine (in the most resistant isolates – HGSA145 and HDG2).

The core genome is also a source of short-term response to antibiotic pressure. Resistance to *β*-lactams can be acquired by substitutions in highly conserved core genes, namely *rpoB* (89, 90). The *rpoB* gene encodes for the DNA-directed RNA polymerase beta chain protein. Both pairs of strains, presented the same nucleotide change at the nucleotidic position 657724: a change from a cytosine (found in the most susceptible strains HU107 and CPS68) to an adenine (in the most resistant strains HGSA145 and HDG2).

The remaining four genes in the core genome affected by non-synonymous mutations and shared by the two pair of strains in study were detected in *sasC* gene which encodes for a LPXTG *S. aureus* surface protein C and *sdrD* gene that encodes for a serine-aspartate repeat-containing protein D, *coa* that encodes a staphylocoagulase precursor, and *ebh* that encodes a very-large surface anchored protein, all of them with a potential role in the virulence of the bacteria.

Besides the affected genes shared by the two pair of strains, other genes related with β-lactam resistance were also affected in each pair of strains, namely *relA, valS* (72)for pair A and *pbpA* and *blaZ* (71) for pair B. The mutations found in *rpoB* in both pairs of strains and in other genes related with β-lactam resistance may be involved in the cefoxitin resistance levels found in the most resistance strains in study, HGSA145 and HDG2.

Of note, in pair A, a non-synonymous mutation was found affecting the *tcaB* gene that encodes for the teicoplanin resistance membrane protein. This could be clinically relevant because teicoplanin is a glycopeptide antibiotic with a spectrum of activity similar to vancomycin and is used for the treatment of serious infections caused by Gram-positive bacteria, including MRSA. It would be interesting to investigate the level of teicoplanin resistance in the two strains of pair A in order to check if the mutation found has a phenotypic consequence.

Some regions among the analyzed genomes presented low or no coverage, being possible that more differences between the two pairs of strains may exist besides the

ones identified. Moreover, mutations affecting the intergenic regions were not analyzed.

Further studies are needed in order to confirm if any of the mutations found in genes that have been already reported as affecting the β-lactam resistance have in fact implications in the phenotypic level of β-lactam resistance in the strains in study.

Study B: Assessment of allelic variation in the *ccrB* locus in MRSA clones

In order to evaluate the allelic variation in *ccrB* genes among MRSA strains belonging to the same genetic lineages, the genomic variability among the *ccrB* genes was evaluated using the sequencing approach described by Oliveira *et al*. in 2006. The *ccrB* variability was evaluated for the successful clonal lineages for which the SCC*mec* stability remains to be determined, *i.e.* for Brazilian (ST239-III), EMRSA15 (ST22-$IV_{h)}$, EMRSA16 (ST36-II), Berlin (ST45-IV), Pediatric (ST5-$IV_{a)}$, USA300 (ST8-IV_a), European (ST80-IV), SW/Pacific (ST30-IV) clones.

The selection of strains was done with the objective to maximize temporal and geographical differences.

Regarding the *ccrB* alleles identified in each clone, we observed the presence of the same allele within EMRSA15 (SCC*mec* IV_h), allele 400; EMRSA16 (SCC*mec* II), allele 200 and European (SCC*mec* $IV_{c\ and\ NT}$) allele 400, suggesting very low mutation rate in the *ccrB* locus among these clonal lineages.

Concerning the others clones in study, (Berlin, Pediatric, USA300, SW/Pacific and Brazilian) besides the presence of different alleles in each clone, the variability was low, two to three different alleles per clone were identified within each clone. In the Berlin clone (SCC*mec* IV) alleles 400 and 401; Pediatric (SCC*mec* IV_a and SCC*mec* VI), alleles 400, 401 and 600; USA300 (SCC*mec* $IV_{a)}$, alleles 400 and 409; SW/Pacific (SCC*mec* IV), alleles 400 and 401 and Brazilian (SCC*mec* III), allele 300 and a new *ccrB* allele.

The results obtained lead us to the conclusion that despite the geographically and the temporal diversity of the strains chosen, there is a high rate of identical *ccrB* allotypes

whiting strains belonging to the same MRSA clone. More studies are needed in order to prove the functionality of the different *ccrB* alleles found and consequently to infer their role in the stabilization of the SCC*mec* cassettes in the successful clonal lineages analyzed.

Chapter V - References

1. **Murray PR, Rosenthal, K.S., Kobayashi, G.S., Pfaller, M.A.** Medical Microbiology. Third ed: Mosby; 1998.

2. **Tzagoloff H, Novick R.** Geometry of cell division in *Staphylococcus aureus*. Journal of Bacteriology. 1977;129(1):343-50.

3. **Pinho MG, Kjos M, Veening JW**. How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. Nature Reviews Microbiology. 2013;11(9):601-14.

4. **Jordan S, Hutchings MI, Mascher T.** Cell envelope stress response in Gram-positive bacteria. FEMS Microbiology Reviews. 2008;32(1):107-46.

5. **van Heijenoort J.** Recent advances in the formation of the bacterial peptidoglycan monomer unit. Natural Product Reports. 2001;18(5):503-19.

6. **Scheffers DJ, Pinho MG.** Bacterial cell wall synthesis: new insights from localization studies. Microbiology and Molecular Biology Reviews : MMBR. 2005;69(4):585-607.

7. **Kloss WE, Bannerman, T.L.** Manual of Clinical Microbiology. 7th ed: American Society for Microbiology; 1999.

8. **Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL.** The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infectious Diseases. 2005;5(12):751-62.

9. **Toshkova K, Annemuller C, Akineden O, Lammler C.** The significance of nasal carriage of *Staphylococcus aureus* as risk factor for human skin infections. FEMS Microbiology Letters. 2001;202(1):17-24.

10. **Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, Yi D, Zhao B.** Skin colonization by *Staphylococcus aureus* in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. British Journal of Dermatology. 2006;155(4):680-7.

11. **Ladhani S, Garbash M.** Staphylococcal skin infections in children: rational drug therapy recommendations. Pediatric Drugs. 2005;7(2):77-102.

12. **Diep BA, Carleton HA, Chang RF**, Sensabaugh GF, Perdreau-Remington F. Roles of 34 virulence genes in the evolution of hospital- and community-associated

strains of methicillin-resistant *Staphylococcus aureus*. The Journal of Infectious Diseases. 2006;193(11):1495-503.

13. **Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K.** Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. Journal of Bacteriology. 2008;190(1):300-10.

14. **Lindsay JA, Holden MT.** *Staphylococcus aureus*: superbug, super genome? Trends in Microbiology. 2004;12(8):378-85.

15. **Lindsay JA, Holden MT**. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Functional and Integrative Genomics. 2006;6(3):186-201.

16. **Holden MT, Lindsay JA, Corton C, Quail MA, Cockfield JD, Pathak S, Batra R, Parkhill J, Bentley SD, Edgeworth JD.** Genome sequence of a recently emerged, highly transmissible, multi-antibiotic- and antiseptic-resistant variant of methicillin-resistant *Staphylococcus aureus*, sequence type 239 (TW). Journal of Bacteriology. 2010;192(3):888-92.

17. **Shittu AO, Udo EE, Lin J.** Insights on Virulence and Antibiotic Resistance: A Review of the Accessory Genome of *Staphylococcus aureus.* Wounds. 2007;19(9):237-44.

18. Penicillin. ACSIHCLDaDo. [cited 2016 06-01-2016]. Available from: http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/flemingpenicillin.html

19. **Skinner D, Keefer, C.S.** Significance of bacteremia caused by *Staphylococcus aureus.* Archives of Internal Medicine. 1941(68):851-75.

20. **Rammelkamp CH, Maxon, T**. Resistance of *Staphylococcus aureus* to the action of penicillin. Proceedings of the Royal Society for Experimental Biolology and Medicine. 1942;5.

21. **Lowy FD**. Antimicrobial resistance: the example of *Staphylococcus aureus*. Journal of Clinical Investigation. 2003;111(9):1265-73.

22. **Jevons MP**. Celbenin resistant staphylococci. British Medical Journal. 1961;1:124.

23. **Parker MT, Jevons MP**. A Survey of Methicillin Resistance in *Staphylococcus aureus*. Postgraduate Medical Journal. 1964;40:SUPPL:170-8.

24. **Wilke MS, Lovering AL, Strynadka NC.** Beta-lactam antibiotic resistance: a current structural perspective. Current Opinion in Microbiology. 2005;8(5):525-33.

25. **Babic M, Hujer AM, Bonomo RA**. What's new in antibiotic resistance? Focus on beta-lactamases. Drug Resistance Updates : reviews and commentaries in antimicrobial and anticancer chemotherapy. 2006;9(3):142-56.

26. **Llarrull LI, Fisher JF, Mobashery S**. Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new beta-lactams that meet the challenge. Antimicrobial Agents and Chemotherapy. 2009;53(10):4051-63.

27. **Chambers HF.** Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clinical Microbiology Reviews. 1997;10(4):781-91.

28. **Reed P, Atilano ML, Alves R, Hoiczyk E, Sher X, Reichmann NT**, **Pereira PM, Roemer T, Filipe SR, Pereira-Leal JB, Ligoxygakis P, Pinho MG.** *Staphylococcus aureus* Survives with a Minimal Peptidoglycan Synthesis Machine but Sacrifices Virulence and Antibiotic Resistance. PLoS Pathogens. 2015;11(5):e1004891.

29. **Komatsuzawa H, Ohta K, Sugai M, Fujiwara T, Glanzmann P, Berger-Bachi B, Suginaka H.** Tn551-mediated insertional inactivation of the *fmtB* gene encoding a cell wall-associated protein abolishes methicillin resistance in *Staphylococcus aureus.* The Journal of Antimicrobial Chemotherapy. 2000;45(4):421-31.

30. **Drawz SM, Bonomo RA**. Three decades of beta-lactamase inhibitors. Clinical Microbiology Reviews. 2010;23(1):160-201.

31. **Lowy FD**. Antimicrobial resistance: the example of *Staphylococcus aureus*. Journal of Clinical Investigation. 2003;111(9):1265-73.

32. **Olsen JE, Christensen H, Aarestrup FM.** Diversity and evolution of *blaZ* from *Staphylococcus aureus* and coagulase-negative staphylococci. The Journal of Antimicrobial Chemotherapy. 2006;57(3):450-60.

33. **Fuda CC, Fisher JF, Mobashery S.** Beta-lactam resistance in *Staphylococcus aureus:* the adaptive resistance of a plastic genome. Cellular and Molecular Life Sciences. 2005;62(22):2617-33.

34. **Ryffel C, Tesch W, Birch-Machin I, Reynolds PE, Barberis-Maino L, Kayser FH, Berger-Bachi B.** Sequence comparison of *mecA* genes isolated from methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Gene. 1990;94(1):137-8.

35. **Ubukata K, Nonoguchi R, Song MD, Matsuhashi M, Konno M**. Homology of *mecA* gene in methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus simulans* to that of *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. 1990;34(1):170-2.

36. **Arede P, Milheirico C, de Lencastre H, Oliveira DC**. The anti-repressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of beta-lactam resistance in MRSA. PLoS Pathogens. 2012;8(7):e1002816.

37. **Livermore DM**. Beta-lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews. 1995;8(4):557-84.

38. **Novick RP**. Staphylococcal penicillinase and the new penicillins. Biochemical Journal. 1962;83:229-35.

39. **Richmond MH**. Purification and Properties of the Exopenicillinase from *Staphylococcus aureus.* Biochemical Journal. 1963;88:452-9.

40. **Milheirico C, Portelinha A, Krippahl L, de Lencastre H, Oliveira DC.** Evidence for a purifying selection acting on the beta-lactamase *locus* in epidemic clones of methicillin-resistant *Staphylococcus aureus*. BMC Microbiology. 2011;11:76.

41. **McKinney TK, Sharma VK, Craig WA, Archer GL**. Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (*mecA*) is corepressed but not coinduced by cognate *mecA* and beta-lactamase regulators. Journal of Bacteriology. 2001;183(23):6862-8.

42. **Hackbarth CJ, Chambers HF.** *blaI* and *blaR1* regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. 1993;37(5):1144-9.

43. **Ryffel C, Kayser FH, Berger-Bachi B**. Correlation between regulation of *mecA* transcription and expression of methicillin resistance in staphylococci. Antimicrobial Agents and Chemotherapy. 1992;36(1):25-31.

44. **Wilke MS, Hills TL, Zhang HZ, Chambers HF, Strynadka NC.** Crystal structures of the Apo and penicillin-acylated forms of the BlaR1 beta-lactam sensor of *Staphylococcus aureus*. The Journal of Biological Chemistry. 2004;279(45):47278-87.

45. **Katayama Y, Ito T, Hiramatsu K**. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. 2000;44(6):1549-55.

46. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrobial Agents and Chemotherapy. 2009;53(12):4961-7.

47. **Chatterjee SS, Otto M**. Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves. Clinical Epidemiology. 2013;5:205-17.

48. **Ito T, Katayama Y, Hiramatsu K.** Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrobial Agents and Chemotherapy. 1999;43(6):1449-58.

49. **Wu Z, Li F, Liu D, Xue H, Zhao X.** A novel type XII staphylococcal cassette chromosome *mec* harboring a new cassette chromosome recombinase, CcrC2. Antimicrobial Agents and Chemotherapy. 2015.

50. **Fluit AC, Carpaij N, Majoor EA, Bonten MJ, Willems RJ.** Shared reservoir of *ccrB* gene sequences between coagulase-negative staphylococci and methicillin-resistant *Staphylococcus aureus*. The Journal of Antimicrobial Chemotherapy. 2013;68(8):1707-13.

51. **Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi N, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanahisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori**

M, Ogasawara N, Hayashi H, Hiramatsu K. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. Lancet. 2001;357(9264):1225-40.

52. **Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K.** Genome and virulence determinants of high virulence community-acquired MRSA. Lancet. 2002;359(9320):1819-27.

53. **Ito T, Okuma K, Ma XX, Yuzawa H, Hiramatsu K.** Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Drug Resistance Updates : reviews and commentaries in antimicrobial and anticancer chemotherapy. 2003;6(1):41-52.

54. **Boundy S, Safo MK, Wang L, Musayev FN, O'Farrell HC, Rife JP, Archer GL .** Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfX*, the gene containing the staphylococcal chromosome cassette *mec* (SCC*mec*) insertion site. The Journal of Biological Chemistry. 2013;288(1):132-40.

55. **Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, Katayama Y, Matsuo M, Kuwahara-Arai K, Hishinuma T, Baba T.** Genomic Basis for Methicillin Resistance in *Staphylococcus aureus*. Infection & Chemotherapy. 2013;45(2):117-36.

56. **Wu SW, de Lencastre H, Tomasz A.** Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*. Journal of Bacteriology. 2001;183(8):2417-24.

57. **Wielders CL, Vriens MR, Brisse S, de Graaf-Miltenburg LA, Troelstra A, Fleer A, Schmitz FJ, Verhoef J, Fluit AC.** Evidence for *in-vivo* transfer of *mecA* DNA between staphylococci. Lancet. 2001;357(9269):1674-5.

58. **Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG.** The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proceedings of the National Academy of Sciences of the United States of America. 2002;99(11):7687-92.

59. **Ito T, Katayama Y, Asada K, Mori N, Tsutsumimoto K, Tiensasitorn C, Hiramatsu K.** Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant

Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2001;45(5):1323-36.

60. **Berglund C, Ito T, Ikeda M, Ma XX, Soderquist B, Hiramatsu K.** Novel type of staphylococcal cassette chromosome *mec* in a methicillin-resistant *Staphylococcus aureus* strain isolated in Sweden. Antimicrobial Agents and Chemotherapy. 2008;52(10):3512-6.

61. **Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K**. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrobial Agents and Chemotherapy. 2004;48(7):2637-51.

62. **Ma XX, Ito T, Tiensasitorn C, Jamklang M, Chongtrakool P, Boyle-Vavra S, Daum RS, Hiramatsu K.** Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. Antimicrobial Agents and Chemotherapy. 2002;46(4):1147-52.

63. **Oliveira DC, Milheirico C, de Lencastre H**. Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCC*mec* type VI. Antimicrobial Agents and Chemotherapy. 2006;50(10):3457-9.

64. **Zhang K, McClure JA, Elsayed S, Conly JM**. Novel staphylococcal cassette chromosome *mec* type, tentatively designated type VIII, harboring class A *mec* and type 4 *ccr* gene complexes in a Canadian epidemic strain of methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. 2009;53(2):531-40.

65. **Deurenberg RH, Stobberingh EE**. The evolution of *Staphylococcus aureus*. Infection, genetics and evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases. 2008;8(6):747-63.

66. **Harris SR, Feil EJ, Holden MT, Quail MA, Nickerson EK, Chantratita N, Gardete S, Tavares A, Day N, Lindsay JA, Edgeworth J, de Lencastre H, Parkhill J,Peacock SJ, Bentley SD.** Evolution of MRSA during hospital transmission and intercontinental spread. Science. 2010;327(5964):469-74.

67. **Wyllie D, Paul J, Crook D**. Waves of trouble: MRSA strain dynamics and assessment of the impact of infection control. The Journal of Antimicrobial Chemotherapy. 2011;66(12):2685-8.

68. **Lowy FD**. *Staphylococcus aureus* infections. The New England Journal of Medicine. 1998;339(8):520-32.

69. **Okuma K, Iwakawa K, Turnidge JD, Grubb WB, Bell JM, O'Brien FG, Coombs GW, Pearman JW, Tenover FC, Kapi M, Tiensasitotn C, Ito T, Hiramatsu K.** Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. Journal of Clinical Microbiology. 2002;40(11):4289-94.

70. **Chambers HF**. The changing epidemiology of *Staphylococcus aureus*? Emerging Infectious Diseases. 2001;7(2):178-82.

71. **Roemer T, Schneider T, Pinho MG.** Auxiliary factors: a chink in the armor of MRSA resistance to beta-lactam antibiotics. Current Opinion in Microbiology. 2013;16(5):538-48.

72. **Dordel J, Kim C, Chung M, Pardos de la Gandara M, Holden MT, Parkhill J, de Lencastre H, Bentley SD, Tomasz A.** Novel determinants of antibiotic resistance: identification of mutated *loci* in highly methicillin-resistant subpopulations of methicillin-resistant *Staphylococcus aureus*. mBio. 2014;5(2):e01000.

73. **Shibata H, Nakano T, Niimi S, Fujii N, Arakaki N, Higuti T.** No relationship exists between PBP 2a amounts expressed in different MRSA strains obtained clinically and their beta-lactam MIC values. Journal of Medical Investigation. 2008;55(3-4):246-53.

74. **Berger-Bachi B, Rohrer S**. Factors influencing methicillin resistance in staphylococci. Archives of Microbiology. 2002;178(3):165-71.

75. **Sabat AJ, Pournaras S, Akkerboom V, Tsakris A, Grundmann H, Friedrich AW.** Whole-genome analysis of an oxacillin-susceptible CC80 *mecA*-positive *Staphylococcus aureus* clinical isolate: insights into the mechanisms of cryptic methicillin resistance. The Journal of Antimicrobial Chemotherapy. 2015;70(11):2956-64.

76. **Tomasz A, Nachman S, Leaf H**. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrobial Agents and Chemotherapy. 1991;35(1):124-9.

77. **Sakoulas G, Gold HS, Venkataraman L, DeGirolami PC, Eliopoulos GM, Qian Q.** Methicillin-resistant *Staphylococcus aureus:* comparison of susceptibility testing methods and analysis of *mecA*-positive susceptible strains. Journal of Clinical Microbiology. 2001;39(11):3946-51.

78. **Dark MJ.** Whole-genome sequencing in bacteriology: state of the art. Journal of Infection and Drug Resistance. 2013;6:115-23.

79. **Harris SR, Cartwright EJ, Torok ME, Holden MT, Brown NM, Ogilvy-Stuart AL, Ellington MJ, Quail MA, Bentley SD, Parkhill J, Peacock SJ.** Whole-genome sequencing for analysis of an outbreak of meticillin-resistant *Staphylococcus aureus*: a descriptive study. Lancet Infectious Diseases. 2013;13(2):130-6.

80. **Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth L, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whihead S, Barrell BG, Spratt BG, Parkhill J.** Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(26):9786-91.

81. **Institute CLS**. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-First Informational Supplement ed2011.

82. **Oliveira DC, de Lencastre H.** Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy. 2002;46(7):2155-61.

83. **Oliveira DC, Milheirico C, Vinga S, de Lencastre H**. Assessment of allelic variation in the *ccrAB locus* in methicillin-resistant *Staphylococcus aureus* clones. The Journal of Antimicrobial Chemotherapy. 2006;58(1):23-30.

84. **Taylor CR**. New revised Clinical and Laboratory Standards Institute Guidelines for Immunohistochemistry and Immunocytochemistry. Applied Immunohistochemistry & Molecular Morphology. 2011;19(4):289-90.

85. **Ochman H, Lawrence JG, Groisman EA**. Lateral gene transfer and the nature of bacterial innovation. Nature. 2000;405(6784):299-304.

86. **Fraser C, Hanage WP, Spratt BG**. Recombination and the nature of bacterial speciation. Science. 2007;315(5811):476-80.

87. **Croll D, McDonald BA**. The accessory genome as a cradle for adaptive evolution in pathogens. PLoS Pathogens. 2012;8(4):e1002608.

88. **Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Renington F.** Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet. 2006;367(9512):731-9.

89. G**ordon NC, Price JR, Cole K, Everitt R, Morgan M, Finney J, Kearns AM, Pichon B, Young B, Wilson DJ, Llewelyn MJ, Paul J, Peto TE, Crook DW, Walker AS, Golubchik T.** Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome sequencing. Journal of Clinical Microbiology. 2014;52(4):1182-91.

90. **Wright GD.** The antibiotic resistome: the nexus of chemical and genetic diversity. Nature Reviews Microbiology. 2007;5(3):175-86.

Chapter VI – Annexes

Annex 1

Supplementary Table 1S - Collection of strains belonging to the Brazilian clone

Strain	Isolation year	Isolation origin	SCC*mec* type	SCC*mec* subtype	*spa*	MLST ST	Oxacillin disk (mm)	Cefoxitin disk (mm)
CHI55	1998	China	III	ND	t037	239	6	8
CHI59	1998	China	III	ND	t037	239	6	6
HGSA142B	2003	Portugal	III	IIIA	t037	239	6	6
HGSA145	2003	Portugal	III	IIIA	t037	ND	9	6
HGSA153	2003	Portugal	III	IIIA	t037	ND	6	6
HGSA154	2003	Portugal	III	IIIA	t037	ND	6	6
HGSA155	2003	Portugal	III	IIIA	t037	ND	6	6
HGSA339	2003	Portugal	III	IIIA	t037	239	6	6
HU42	1995	Hungary	III	ND	t787	ND	6	10
HU103	1996	Hungary	III	ND	t787	ND	15	25
TAW3	1998	Taiwan	III	ND	t037	ND	6	6
TAW5	1998	Taiwan	III	IIIA	t037	ND	7,5	6
TAW27	1998	Taiwan	III	IIIA	t037	ND	6	7
TAW75	1998	Taiwan	III	ND	t037	ND	6	6
TAW97	1998	Taiwan	III	IIIA	t037	239	6	6
TAW217	1998	Taiwan	III	ND	t037	ND	8	8
TAW219	1998	Taiwan	III	ND	t037	ND	6	6
HU2	1996	Hungary	III	ND	t989	239	14	14
HU16	1996	Hungary	III	ND	t787	ND	7	10
HU107	1996	Hungary	III	ND	t037	ND	6	12
HU139	1994	Hungary	III	ND	t989	ND	19	23
HU176	1995	Hungary	III	ND	t037	ND	6	14
HUC343	2006	Portugal	III	IIIA	t037	ND	7	6
SAMS113	NA	Portugal	III	ND	t037	ND	6	6
SAMS363	NA	Portugal	III	ND	t037	ND	6	6
TUR1	1996	Turkey	III	ND	t030	239	6	8
TUR4	1996	Turkey	III	ND	t030	239	6	9
TUR9	1995	Turkey	III	ND	t030	239	6	8
TUR27	1996	Turkey	III	ND	t030	239	6	7,5
CPS 32	1985	Portugal	III	ND	ND	ND	6	8
CPS 33	1985	Portugal	III	ND	ND	ND	6	7
CPS 36	1985	Portugal	III	ND	ND	ND	6	8
CPS 41	1985	Portugal	III	ND	ND	ND	6	7
CPS 42	1985	Portugal	III	ND	ND	ND	6	6
CPS 43	1985	Portugal	III	ND	ND	ND	6	6
CPS 77	1985	Portugal	III	ND	ND	ND	6	9
C335	2009	Hungary	III	ND	t030	ND	6	8
R35	1987	USA	III	ND	t037	239	6	10
ANS46	1982	Australia	III	ND	t037	239	6	9

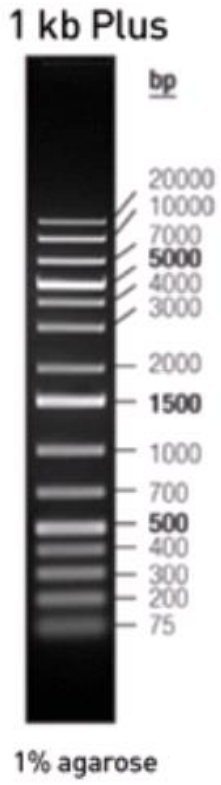
Supplementary Table 1S - Collection of strains belonging to the Brazilian clone (continuation)

Strain	Isolation year	Isolation origin	SCC*mec* type	SCC*mec* subtype	*spa*	MLST ST	Oxacillin disk (mm)	Cefoxitin disk (mm)
HU25	1993	Brazil	III	IIIA	t138	239	6	6
HSJ216	1997	Portugal	III	IIIA	t037	239	7	6
HUSA304	1993	Hungary	III	III	t1053	239	14	14
HU106	1996	Hungary	III	ND	t538	239	6	13
BK2421	1996	USA	III	IIIA	t037	239	7	8
LHH1	1994	USA	III	ND	t037	239	7	9
HDG2	1993	Portugal	III	IIIB	t421	239	6	6
HSA10	1992	Portugal	III	ND	t421	239	6	10
HGSA9	1997	Portugal	III	ND	NT	239	9	8
HGSA15	1994	Portugal	III	IIIA	t037	239	6	9
HGSA57	1995	Portugal	III	IIIA	t037	239	8	6
GRE4	1998	Greece	III	IIIA	t037	239	6	6
GRE18	1998	Greece	III	ND	t037	239	10	7
GRE108	1998	Greece	III	ND	t461	239	6	8
GRE317	1999	Greece	III	ND	t138	239	6	7
URU34	1997	Uruguay	III	ND	t037	239	6	6
URU110	1998	Uruguay	III	ND	t037	239	6	6
CHL1	1997	Chile	III	ND	t037	239	6	6
CHL151	1998	Chile	III	ND	t037	239	6	6
BRA36	1997	Brazil	III	ND	t037	239	6	6
AGT1	1997	Argentina	III	ND	t037	239	6	6
AGT9	1997	Argentina	III	ND	t037	239	6	8
AGT67	1997	Argentina	III	ND	t037	239	6	6
AGT120	1998	Argentina	III	ND	t037	239	6	11
CPS22	1985	Portugal	III	ND	t421	239	6	10
CPS68	1985	Portugal	III	ND	t421	239	6	13
HU294	2003	Hungary	III	ND	t538	239	6	8
HUR4	1997	Hungary	III	ND	t787	239	6	11
HU272	2002	Hungary	III	ND	t787	239	6	12
HU109	1996	Hungary	III	ND	t984	239	6	8
HU180	1996	Hungary	III	ND	t984	ND	6	7
HU219	1995	Hungary	III	ND	t984	ND	6	9
HU12	1996	Hungary	III	ND	t984	ND	6	8
HU276	1995	Hungary	III	ND	t1053	239	6	13

Note: The following CLSI breakpoints for testing the susceptibility of *S. aureus* were used: oxacillin MIC susceptible (S) ≥ 13 mm, intermediate (I) = 11-12 mm, resistant (R) ≤ 10 mm; cefoxitin MIC susceptible (S) ≥ 22 mm and resistant (R) ≤ 21 mm.

Annex 2

1 kb DNA Ladder Plus (Thermo Scientific, Fermentas)



Annex 3

Extraction of DNA by the Qiagen DNeasy Blood & Tissue Kit

(Optimized protocol from QIAGEN, Ambion Inc, Austin, Texas, U.S)

Day 1:

1) Inoculate 5 ml of TSB broth. Incubate o/n at 37°C (16-18h with aeration).

Day 2:

2) Harvest 500 µl of the culture, centrifuge for 5 min at 8000 rpm. Discard supernatant.
3) Add 200 µl LP1 (lysis buffer) plus 3 µl RNase (10 mg/ml), 10 µl lysostaphin (10 mg/ml) and 5 µl of lysozyme (20 mg/ml) and ressuspend the bacterial pellet.
4) Incubate 1 hour at 37°C.
5) Add 25 µl proteinase K (Kit) and 200 µl Buffer AL (without ethanol); mix gently.
6) Incubate for 60 min at 56°C.
7) Add 200 µl of 96% ethanol; mix gently.
8) Transfer the mixture to the DNeasy column. Note: A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column.
9) Centrifuge for 1 min, at 8000 rpm. Discard the flow-through and collection tube.
10) Place the spin column in a new 2 ml collection tube. Add 200 µl Buffer AW1.
11) Centrifuge for 1 min, at 8000 rpm. Discard the flow-through and collection tube.
12) Transfer the spin column to a new 2 ml collection tube. Add 200 µl Buffer AW2.
13) Centrifuge for 3 min, at 14000 rpm. Discard the flow-through and collection tube.
14) Transfer the spin column to a new 1.5 ml eppendorf tube and add 50 µl Tris-HCl 5 mM (pH 8 - 8.5) to the center of the spin column membrane.
15) Incubate for 1 min at room temperature (15 - 25°C) and centrifuge for 1 min at 8000 rpm. (Keep the column).
16) Measure the DNA concentration in nanodrop. If necessary repeat steps 14-16, using the same column.

Annex 4

Extraction of DNA by the boiling method

Day 1:

1) Inoculate 5 ml of TSB broth. Incubate for 16-18h with aeration.

Day 2:

2) Harvest 500 µl of the culture, centrifuge for 2 min at 13000 rpm. Discard supernatant.
3) Add 50 µl TE 1X and ressuspend the bacterial pellet.
4) Add 1 µl lysostaphin (10 mg/ml).
5) Incubate 30 min at 37°C.
6) Incubate 15 min at 95°C.
7) Add 150 µl of water and vortex.
8) Centrifuge for 5 min, at 13000 rpm.
9) Collect supernatant (about 150 µl). Store at -20°C.

Annex 5

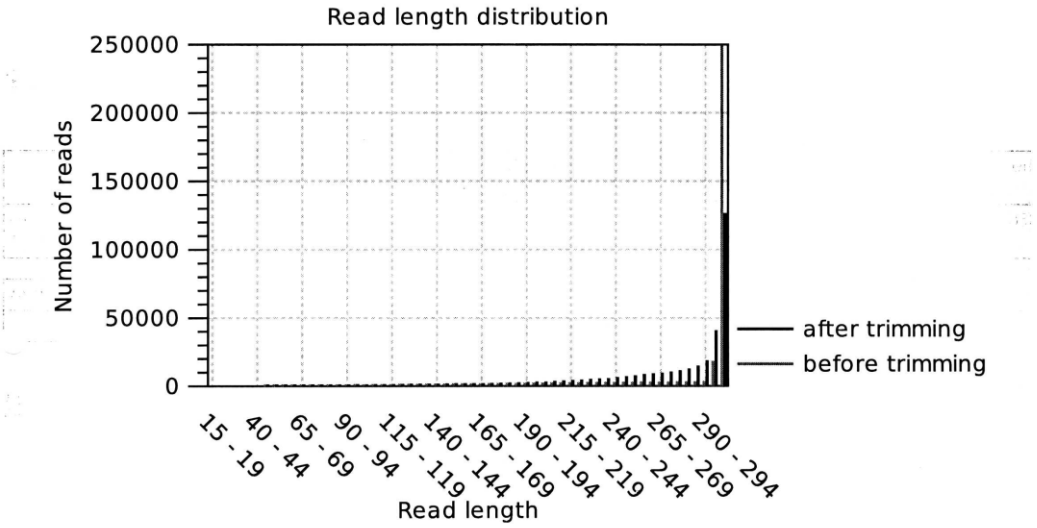
HU107 Trim summary

1. Trim summary

Name	Number of reads	Avg.length	Number of reads after trim	Percentage trimmed
HU107_S26_L001_R1_001 (paired)	376,548	270.2	376,291	99.93%

Avg.length after trim
259.7

2. Read length before / after trimming



3. Trim settings

- Removal of low quality sequence. (limit = 0.05).
- Removal of ambiguous nucleotides: maximal 2 nucleotides allowed.
- Removal of sequences on length: minimum length 15 nucleotides.

Annex 6

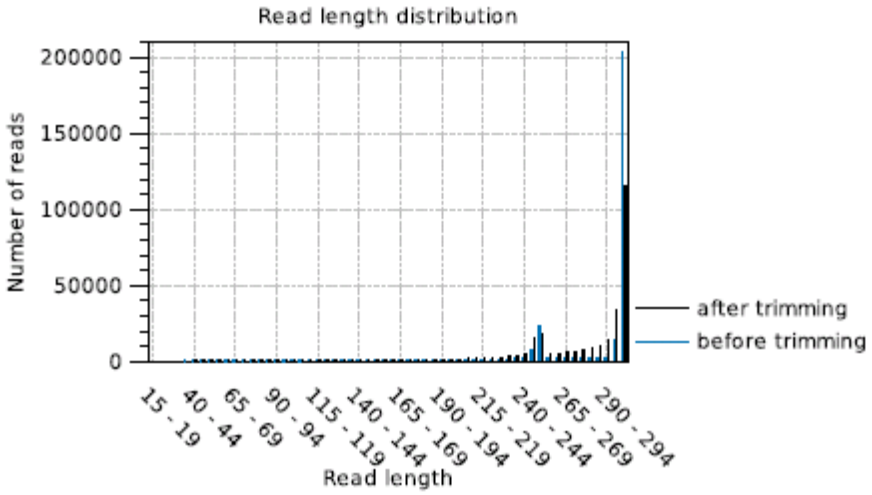
HGSA145 Trim summary

1. Trim summary

Name	Number of reads	Avg.length	Number of reads after trim	Percentage trimmed
HGSA145_S11_L001_R1_001 (paired)	298,644	272.5	298,434	99.93%
HGSA145_S13_L001_R1_001 (paired)	33,578	235.0	33,555	99.93%

Avg.length after trim
264.4
233.4

2. Read length before / after trimming



3. Trim settings

- Removal of low quality sequence. (limit = 0.05).
- Removal of ambiguous nucleotides: maximal 2 nucleotides allowed.
- Removal of sequences on length: minimum length 15 nucleotides.

Annex 7

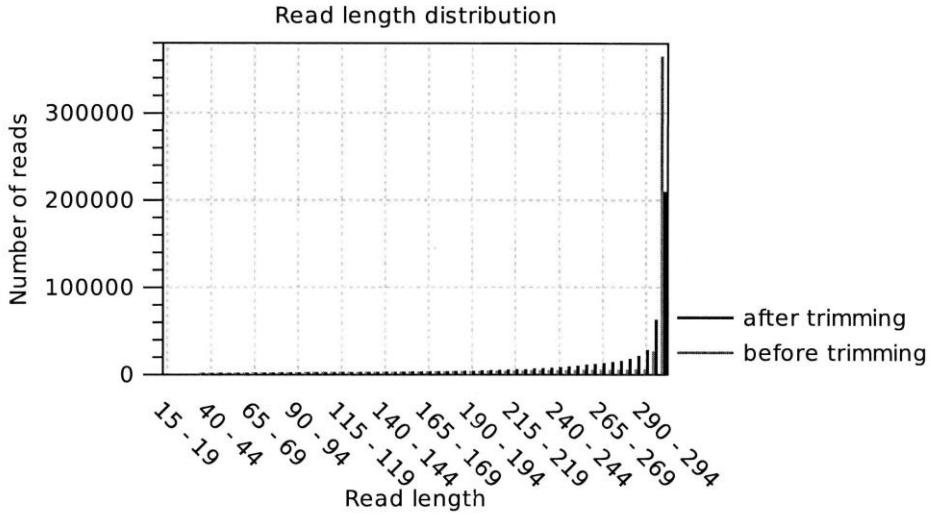
CPS68 Trim summary

1. Trim summary

Name	Number of reads	Avg.length	Number of reads after trim	Percentage trimmed
CPS68_S18_L001_R1_001 (paired)	595,482	262.4	594,861	99.9%

Avg.length after trim
255.4

2. Read length before / after trimming



3. Trim settings

- Removal of low quality sequence. (limit = 0.05).
- Removal of ambiguous nucleotides: maximal 2 nucleotides allowed.
- Removal of sequences on length: minimum length 15 nucleotides.

Annex 8

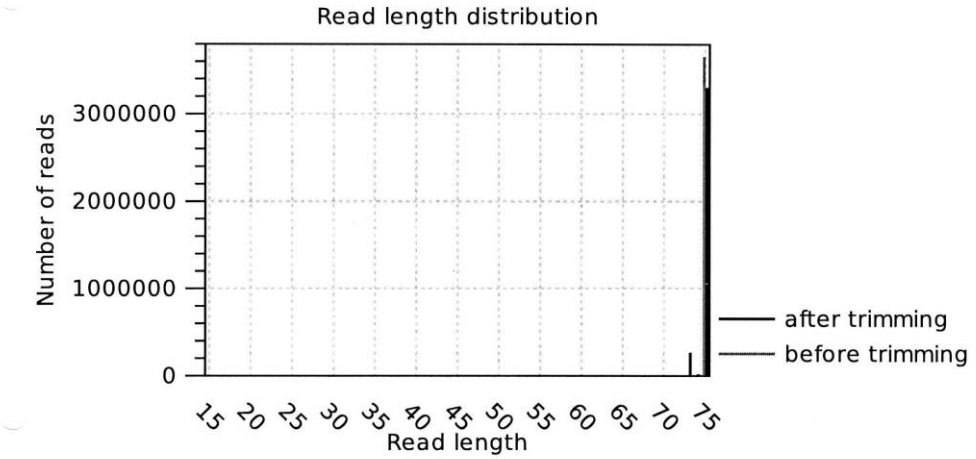
HDG2 Trim summary

1. Trim summary

Name	Number of reads	Avg.length	Number of reads after trim	Percentage trimmed
HDG2_1 (paired)	3,657,232	75.0	3,653,828	99.91%

Avg.length after trim
74.5

2. Read length before / after trimming



3. Trim settings

- Removal of low quality sequence. (limit = 0.05).
- Removal of ambiguous nucleotides: maximal 2 nucleotides allowed.
- Removal of sequences on length: minimum length 15 nucleotides.