Chapter 2

Typification Methods and Molecular Epidemiology of *Staphylococcus aureus* with Methicillin Resistance

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Abstract

Recent interest in the study of *Staphylococcus aureus* derives from the high frequency of antibiotic-resistant strains that cause frequent outbreaks of infection, especially methicillin-resistant *S. aureus* (MRSA). The objective of this chapter was to study the population genetic structure and the origin of MRSA isolation. Classification of staphylococcal cassette chromosome mec (SCCmec) is the most important method to identify and define the *S. aureus* methicillin-resistant clonal nature. Molecular epidemiological studies have demonstrated dissemination patterns of few strains which are responsible for the important worldwide problem. There is a predominance of pandemic clones of MRSA associated to hospital-acquired infections (HA-MRSA) which has been replaced today by community-acquired strains (CA-MRSA). Understanding the epidemiology and clonality of *S. aureus* infections has important implications for future efforts to control of the emergence of multidrug-resistant strains and the spread of clones resistant and sensible to methicillin.

Keywords: methicillin-resistant *S. aureus*, clonal complex, molecular epidemiology, classification, typing

1. Introduction

Staphylococcus aureus is one of the most common pathogenic organisms responsible for a wide variety of infectious syndromes [1, 2]. Significant increase in the prevalence and emergence of methicillin-resistant *S. aureus* (MRSA) is a serious public health concern and has a dramatic negative impact on medical practices [3, 4]. Therefore, identification of MRSA strains is important for both clinical and epidemiological implications.

On the other hand, it is important to carry out the typing of *S. aureus* to defining the occurrence of an epidemic, monitoring the transmission of the organism among carriers, contributes to the evaluation of nosocomial infection outbreaks, recurrent infection and the use of the appropriate measures in a local environment. In addition, determining the origins of these strains can help in delineating their circulation among different environments [5, 6].

Different genotypic and phenotypic methods have been developed for this purpose. However, each method has its own advantages and disadvantages, so the optimal method of bacterial strain typing depends on the objectives of data collection and available resources [7–9]. Thus, techniques with high discriminatory power with the ability to distinguish between epidemiologically unrelated bacterial strains are adequate for carrying out locally restricted epidemiological studies or epidemic outbreaks. While, the sequence-based techniques that analyze more stable genetic markers are more appropriate for recognizing ancestral relationships between the bacterial strains [9].

In this chapter, we expose the methods of detection and typing of *S. aureus* and MRSA isolations, through which progress has been made in understanding the molecular epidemiology of the bacterium.

2. Identification of *S. aureus*

The high pathogenicity of *S. aureus* causes frequent nosocomial and community infections, so its isolation and rapid identification is extremely important for timely treatment [1, 2]. The diagnosis of diseases caused by *S. aureus* should be based first of all on the clinical picture and then confirm with a culture where it is isolated [6, 10].

Gram staining of the colony and tests for the production of catalase and coagulase are the ideal techniques that allow the rapid identification of coagulase-positive *S. aureus* [11, 12]. Another very useful test for its identification is the production of thermostable deoxyribonuclease [12].

2.1. Latex agglutination test

S. aureus produces two forms of coagulase: bound coagulase, or "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected using a tube coagulase test. Hemagglutination test with fibrinogen-sensitized sheep erythrocytes is used for the detection of clumping factor.

Also slide agglutination test with plasma-coated latex is used for the simultaneous detection of clumping factor and protein A. In principle, plasma contains fibrinogen, which has the capacity to bind to clumping factor, and immunoglobulin, which has the capacity to bind to protein A through its Fc fragment. Hence, the presence of either clumping factor or protein A on the bacterial cell results in co-agglutination of cells and latex particles [13].

There are variants of the agglutination tests that use different surface antigens, specific for *S. aureus*, which contributes to an increase in the sensitivity of the tests, especially for some *S. aureus* isolates that produce relatively small amounts of coagulase or protein A [14].

On the other hand, the techniques based on the molecular identification of *S. aureus* like fluorescent in situ hybridization (FISH) use artificial probes labeled with fluorescent molecules and specific for *S. aureus* are applied in order to differentiate this species [15]. Molecular tests based on the PCR method, which demonstrate the genes which code nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), *fem*A, *fem*B, sa442, 16SrRNA and fibrinogen-binding proteins [16–18].

3. Detection of MRSA isolates

The fact that methicillin resistance is undoubtedly related to the importance of the *mec*A gene, makes it possible to create molecular tests relatively quickly for definite proof of MRSA.

S. aureus acquires methicillin resistance through *mec*A gene that is responsible for the synthesis of a 78-kDa protein, called penicillin-binding protein 2a (PBP2a). PBP2a substitutes other PBPs, that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains, but its active site blocks binding of all β-lactams but allows the transpeptidation [19, 20].

The *mec*A gene is regulated by the repressor MecI and the trans-membrane β-lactam-sensing signal transducer MecRI, both of which are transcribed divergently. However, in the absence of a β-lactam antibiotic, MecI represses the transcription of both *mec*A and *mec*RI-*mec*I. In the presence of a β-lactam antibiotic, MecRI is cleaved autocatalytically, and a metallo-protease domain, which is located in the cytoplasmic part of MecRI, becomes active. The metallo-protease cleaves MecI bound to the operator region of *mec*A, which allows transcription of *mec*A and subsequent production of PBP2a [19].

The *mec*A gene is part of a genomic island designated staphylococcal cassette chromosome mec (*SCCmec*) [21].

SCCmec elements integrate sequence at the bacterial chromosomal attachment site (*attBscc*) that is located near the origin of replication, at the 3′ end of an open reading frame of unknown function, termed *orfX*, well conserved among both MRSA and MSSA strains [21–23].

The attachment site contains a core 15-bp sequence, called the integration site sequence (ISS) that is necessary for ccr-mediated recombination [21]. *SCCmec* integrated into the chromosome consists of *mec* complex, composed of *mec*A operon, *ccr* gene complex, composed of cassette chromosome recombinase (*ccr*) gene(s) and three regions bordering the ccr and mec complexes, designated as joining (J) regions, that is, with composition presented as follow: (*orf*X)J3-*mec*-J2-*ccr*-J1 [21, 22].

The 2.1-kb *mec*A gene is regulated by the repressor MecI and MecRI that are transcribed divergently. In the absence of a β-lactam antibiotic, MecI represses the transcription of both *mec*A and *mec*RI-*mec*I. In the presence of a β-lactam antibiotic, MecRI is activated by autolytic cleavage and cleaves MecI bound to the operator region of *mec*A, which allows transcription of *mec*A and subsequent production of PBP2a [21–23]. Both *mec*I and *mec*RI can be truncated by insertion sequences IS431 or IS1272, and these results in derepression of the *mec*A gene [24].

There is a *mec*A homolog, termed *mec*C, which is only ~69% identical to conventional *mec*A at the DNA level, and the encoded PBP2a/2′ is ~63% identical at the amino acid level. Similar

to conventional *mec*A, *mec*C is located within a *SCCmec* element inserted into the 3′ region of orfX but it had divergent ccrA and ccrB recombinases (belonging to the ccrA1 and ccrB3 groups and representing a novel combination of recombinase groups designated type 8 ccr), divergent *mec*A regulatory genes (mecI/mecR) and the absence of one of the three joining regions (J3) that are normally present [25].

In the identification of MRSA, MRSA Screen Latex agglutination test is a slide agglutination assay to detect penicillin-binding protein (PBP2a) from isolates of Staphylococci. The tool contains latex particles sensitized with a monoclonal antibody against PBP2a [26]. The Cefoxitin Disc Diffusion and Oxacillin Agar Screen developed on Muller Hinton agar plates are the phenotypic tests used routinely [27–29]. Methods based on detection of the *mec*A gene, the PCR method are also used in many laboratories [30, 31].

4. Typing of *S. aureus*

Everyday, the techniques of bacterial molecular typing become more available. Optimal typeability, a high degree of reproducibility, adequate stability and unprecedented resolving power characterize the "gold standard" typing technique [8].

4.1. Phenotypic methods

The conventional methods used for the typing of *S. aureus* and especially of the MRSA strains emerged in the 1950s and 1960s, all being phenotypic methods, among these methods, biotype, serotyping, fagotipage and resistograms (resistance to chemicals and dyes) were highlighted [8, 9]. In the case of resistograms, ethidium bromide, cadmium nitrate, phenyl mercuric acetate and mercuric chloride have been used on the basis of the susceptibility pattern produced.

4.2. Serotyping

Serotyping is based on fact that strains of same species can differ in the antigenic determinants expressed on the cell surface such as lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella and fimbriae exhibit antigenic variations. Strains differentiated by antigenic differences are known as 'serotypes'.

Serotyping of capsular polysaccharides in *S. aureus* has allowed to establish a total of 11 capsular types, but 85–90% of clinical isolates belong to just two of them. For example, in SARM only serotype 5 or 8 is detected.

This method has limited application in epidemiological studies because a large number of unrelated isolates belong to a small number of capsular serotypes [32].

4.3. Phage typing

Strains can be characterized by their pattern of resistance or susceptibility to a standard set of bacteriophages. This relies on the presence or absence of particular receptors on the bacterial

surface that are used by the virus to bind to the bacterial wall. This method is used to type isolates of *S. aureus* and is referred as 'phage types' and was standardized by the International Subcommittee on Phage Typing of Staphylococci [33].

Human strains of *S. aureus* are classified according to their susceptibility to a set of set of 23 phages (group I—29, 52, 52A, 79 and 80; group II—3A, 3C, 55 and 71; group III—6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85; group V—94 and 96; not classified—81 and 95) internationally accepted for typing. The technique requires maintenance of biologically active phages and is available only at reference centers. This technique has been reported to be valuable in the identification of known epidemic strains among endemic strains and is preferred as first line approach in epidemiological investigation of MRSA strains [32]. Phagotyping also has limited application since a significant number of isolates are not susceptible to bacteriophages and it is not possible to apply this method to them [33].

4.4. Biotyping

Biotyping is a rapid and inexpensive method that makes use of the pattern of metabolic activities expressed by an isolate, colonial morphology and environmental tolerances and strains are referred to as "biotypes".

Devriese proposed a simplified biotyping system for the typing of *S. aureus* strains on the basis of the evaluation of synthesis of fibrinolysin and β-hemolysin, coagulation of bovine plasma and type of growth on medium containing crystal violet [34]. This method allows to differentiate *S. aureus* isolates from host specific (HS) ecovars: human, bovine, ovine and poultry biotypes; the strains which could not be classified into any of these biotypes on the basis of their properties were referred to as non-host-specific (NHS).

In the 1990s, Isigidi et al. described a new biotype, P-like pA+ (poultry-like protein A positive), and was tentatively designated as an "abattoir" biotype [35]. The introduction of an additional biochemical test, protein A production permitted showed typical properties of the poultry biotype but differed from it in terms of the synthesis of protein A. This biotype was initially described solely in meat products and meat industry workers. In 2016, Piechowicz and Garbacs, revealed that the P-like pA+ biotype strains can be also present in hospitalized patients and extra-hospital carriers with greater genetic variability [36].

This method has been useful in tracing the origin of *S. aureus* isolates in food animal and food industry and the probable source of contamination of foods by *S. aureus*. Kitai et al. showed that retail raw chicken meat in Japan is frequently contaminated with *S. aureus* strains belonging to the human and poultry biotypes [37].

Hakimi et al. showed that different animal ecovars were characterized among human and bovine raw milk isolates, confirm the possibility of the transmission of *S. aureus* strains among humans and different animal species, and this can be very important, especially when such strains carry antibiotic resistance genes [38].

Hennekinne et al. investigated the genotypic discrimination between *S. aureus* strains assigned to different biotypes with PFGE patterns showing a strong correlation between

pulsotypes and biotypes, and confirm the abattoir biotype as an individual group [39]. However, strain discrimination is limited, variation in gene expression, due mainly to point mutations is the most common reason for isolates that represent single strain to differ in one or more biochemical reactions.

4.5. Antimicrobial susceptibility typing (antibiogram)

Phenotyping methods also include examination of susceptibility to antimicrobes, which has the practical value in recommending treatment for the infection and as a strategy in the control of resistance to antibiotics [28, 29].

A common method for the detection of MRSA employs the technique of diffusion in hypersaline Mueller Hinton agar, with a disc of 1 μg of oxacillin, incubating at 35°C for 24–48 h (halo inhibition ≤10 mm) [40] or the study of the minimum inhibitory concentration (MIC) by means of an E-test with oxacillin strip. Additionally, it has been demonstrated that cefoxitin (cefamycin) in vitro, induces the production of PBP2a in strains of sensitive methicillin *S. aureus* [27]; therefore, the disc diffusion method using cefoxitin (FOX 30 μg) has proven to be a good assay for the detection of low level resistance to oxacillin in strains of *S. aureus*. Currently, the cefoxitin disc is used as a substitute for oxacillin for the phenotypic detection of MRSA strains [29].

Antibiogram typing profiles or antibiotypes involves comparison of susceptibilities of isolates to a range of antibiotics. Isolates differing in their susceptibilities are considered as different strains. An unusual pattern of antibiotic resistance among isolates from multiple patients is considered as an indication of an outbreak [41].

Antibiotic susceptibility patterns has been the main typing tool in many hospital outbreaks since the technique is widely available and standardized. With the use of the antibiogram, it has been shown that the pattern of susceptibility to antibiotics varies according with time and geographical location [42]. However, antibiotic resistance patterns are also, to some extent influenced by the local environment, selective antibiotic pressure, acquisition and loss of plasmids carrying resistance genes and various other genetic mechanisms.

One way to optimize the antibiotype to evaluate the clonal relationship between two bacteria is given by the quantitative antibiogram. This mathematical technique proposed for Giacca et al. is based on disc zone sizes, in order to assess the probability of two or more clinical isolates to be the same strain [42]. Method uses the comparison of the diameters of the inhibition rings in the disc diffusion technique (Kirby Bauer) [41]. Antimicrobials are selected with greater variation for the strain under study, to allow better discrimination. The result of the summation of the inhibition zones of a bacterial isolation is evaluated and compared with the other isolation by using a coefficient of similarity.

Similarity of strains is reported in a dendrogram, in which strains are successively fused. Strains that share a common susceptibility pattern are considered a "cluster" [42].

Although useful as a screening method for detecting certain resistance profiles and for selecting potentially useful therapeutic agents, conventional antimicrobial susceptibility testing methods are insensitive tools for tracing the spread of individual strains within a hospital or region [8, 9].

4.6. Molecular typing techniques of *S. aureus*

In order to examine more thoroughly the molecular evolution of *S. aureus*, especially of MRSA and its spread in world terms, several molecular typing techniques have been developed [5, 6, 8, 9]. These methods involve the study of the microbial DNA, the chromosome and plasmid, their composition, homology and presence or absence of specific genes. These techniques are more frequently applied and better appreciated than the phenotypically oriented approaches in taxonomy, epidemiology and evolutionary studies that have enhanced our understanding of disease epidemiology and provided insight into the evolution of bacterial pathogens [5].

4.7. Plasmid profile analysis

Plasmid analysis was the first molecular technique used for epidemiological investigation of MRSA and MSSA [43].

In this technique, the isolates are differentiated according to the number and sizes of plasmids carried by an isolate, but its reproducibility suffers due to the existence of plasmids in different molecular forms such as supercoiled, nicked or linear, each of which migrates differently on electrophoresis.

The plasmids contain resistant genes against a number of antimicrobial agents, so it has been useful to assess the relatedness of individual clinical isolates of *S. aureus*, in the epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance [44].

Agbagwa and Jirigwa determined the antibiotic-resistant pattern and plasmid profile of *S. aureus* obtained from wound swabs and found similar antibiotic resistance pattern, while different plasmid sizes was observed in the isolates [45]. Jaran also found no direct correlation between the patterns of antibiotic resistance and plasmid profiles in clinical isolates of *S. aureus* in hospitals of Saudi Arabia [46]. This disparity can be due to R-plasmids of different sizes which are also responsible for the presence of multiple resistances.

The technique has not been found to be very useful for the investigation of outbreak infections because the plasmids can be spontaneously lost or readily acquired, related strains can exhibit different plasmid profiles. Also, certain genes are contained in transposons that can be readily acquired or deleted. Some isolates may lack plasmids and will not be typeable by this method [44, 45].

4.8. Chromosomal DNA analysis

4.8.1. Ribotyping

Methods designed to recognize restriction fragment length polymorphisms (RFLP) using a variety of gene probes, including rRNA genes (ribotyping) and insertion sequences. The probes generally used are either labeled with radioisotopes or are biotinylated. In this technique, the choice of restriction enzyme used to cleave the genomic DNA, as well as the probes, is crucial. Restriction enzyme *Eco*R1 has been found to be comparatively more useful than other enzymes in producing a good number of bands [47].

The southern blot hybridization of MRSA fragments after RFLP may contain genes specific for staphylococcus in the form of a probe, including the *mec*, transposon Tn554, *agr*, *aph*(2″)-*aac*(6′) (gene resistance to aminoglycoside).

4.8.2. Pulsed-field gel electrophoresis (PFGE)

PFGE is a technique based on digestion of purified chromosomal DNA with restriction enzyme *Sma*I, generating large fragments of DNA that are separated in agarose molds and detection of fragments by PFGE. Migration of large DNA fragments (10–800 kbp) through the electrophoresis gel is realized by use of an electrical field which changes direction over graded time intervals, so minimizing the overlapping of fragments [47, 48]. The obtained PFGE patterns are evaluated with the Dice coefficient and unweighted pair-group matching analysis (UPGMA) settings, according to the criteria described by Tenover et al. [49]. For the application of these criteria, it will be required that the digestion with the enzyme generates a minimum of 10 bands.

In the USA, a national PFGE-based typing system for *S. aureus*, designated as pulsed-field types USA100 through USA1200 that has been an important tool to facilitate the exchange of PFGE strain typing data and epidemiologic information among reference laboratories has been established [50].

4.8.3. Polymerase chain reaction (PCR)-based typing methods

To facilitate the process of the analysis of *S. aureus* isolates, polymerase chain reaction (PCR)-based typing methods have been developed for their simplicity and the obtaining of fast results. With this technique, it is possible to generate DNA profiles that can be analyzed by gel electrophoresis or DNA sequence analysis [51].

4.8.4. PCR-restriction fragment length polymorphisms (PCR-RFLP)

This typing technique involves the amplification of a defined fragment of DNA and subsequent digestion of the amplified product with a restriction enzyme. Variations in the number and sizes of the fragments detected are referred to as restriction fragment length polymorphism (PCR-RFLP). These fragments are separated on agarose gel electrophoresis and strains can be characterized by their restriction profiles [5].

PCR-RFLP of genes coding for two species-specific proteins, coagulase (*coa*) and staphylococcal protein A (*spa*), have been used to discriminate MRSA strains [8, 52].

4.9. DNA sequence analysis-based typing methods

DNA sequence analysis is an objective genotyping method as the genetic code is highly portable, easily stored and can be analyzed in a relational database [5, 8].

4.9.1. Multilocus sequence typing (MLST)

MLST is a well-established method to study bacterial populations exhibiting sufficient nucleotide diversity in a small number of genomic loci [53].

Due to the specific characteristics of *S. aureus*, it is very suitable to follow clonal evolution of MRSA and MSSA, monitoring genetic changes over long periods of time and in different geographical areas, which has allowed to have a global epidemiological view of the bacterium [54].

The method is based on nucleotide sequences analysis of 0.5-kb fragments from seven housekeeping genes of *S. aureus*: *arcc*, *aroe*, *glpF*, *gmk*, *pta*, *tpi* and yqil. They code the following enzymes, respectively: carbamate kinase, shikimate dehydrogenase, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triosephosphate isomerase and acetyl-coenzyme A acetyltransferase [55]. Since mutations accumulate slowly in housekeeping genes, the MLST scheme is used to delineate clusters of closely related strains.

The sequencing of each gene allows obtaining the allelic profile or sequence type (ST) profile, which are given by the alleles of the seven genes.

The Iberian clone is the most frequent with a MLST profile 3-3-1-12-4-4-16, and belongs to ST247 (www.mlst.net).

The analysis of the MRSA structure is based on the determination of the ST and the *SCCmec* type and is grouped into clonal complexes (cc). Isolates of *S. aureus* are assigned to the same clonal complex when 5 of 7 genes have identical sequences. This analysis is carried out using the 'eBURST', a computer program (based on repeated sequences), developed at the University of Bath in the UK that detail how MRSA spread [56]. Databases containing MLST and associated data from hundreds or thousands of isolates can be accessed via the internet (http://www.mlst.net/ and http://pubmlst.org/) [57].

MLST has provided numerous insights into the epidemiology and population genetics of bacteria and is an excellent tool for investigating the clonal evolution of MRSA. However, MLST is not suitable to characterize the differences in strains within an outbreak as its power to resolve small evolutionary differences is too low. In addition, the costs of sequencing currently limit their routine uses for most epidemiological studies [53–55].

4.9.2. Single-locus sequence typing

Single-locus sequence typing (SLST) is used to compare sequence variation of a single target gene. The genes selected are usually of short sequence repeat (SSR) regions that are sufficiently polymorphic to provide useful resolution. The technique is simple, rapid and highly reproducible [5, 8].

4.9.3. Typing coagulase (coa)

The coagulase gene amplification discriminatory power relies on the heterogeneity of the region containing the 81 bp tandem repeats at the 3'coding region of the coagulase gene which differs both in the number of tandem repeats and the location of *Alu*I and *Hae*III restriction sites among different isolates [52]. Variations in the sequence of genes coding for coagulase (*coa*) showed a good correlation with PFGE typing.

4.9.4. Spa typing

The *spa* gene contains three distinct regions: Fc, X and C [52]. *Spa* typing is a single-locus typing based on sequencing of short sequence repeat (*ssr*) regions of the polymorphic X region of the protein A gene (*spa*) of *S. aureus* [60]. The polymorphic X region consists of up to 12 units each with a length of 24-bp variable-number tandem repeat (VNTR) within the 3′ coding region. The composition of the repeating fragments is presented in letters, in that a group of fragments in a certain isolate comprises the "*spa* repeat" code. The repeating fragments are also marked by a number, in view of their large number and for easier data processing.

Isolates are assigned to particular *spa* types using the *spa* typing website (http://www.spaserver.ridom.de). Several studies have demonstrated that *spa* typing is highly discriminatory, and useful in both local and global epidemiological studies [58].

In addition to its use as a marker, the number of repeats in the region X of *spa* has been related to the dissemination potential of MRSA, with higher numbers of repeats associated with higher epidemic capability; it detects genetic microvariations and may be used in phylogenetic studies, where genetic macrovariations are key [58].

4.9.5. SCCmec typing

The first *SCCmec* element was identified in Japanese *S. aureus* strain and shortly after two additional *SCCmec* were determined; these three *SCCmec* elements were classified as types I–III [23, 61]. Subsequently, two other *SCCmec* were described: *SCCmec*IV [59] and *SCCmec*V [60].

Currently, 11 *SCCmec* types are known: *SCCmec*VI, *SCCmec*VII, *SCCmec*VIII, *SCCmec*IX, *SCCmec*X, *SCCmec*XI [61–63].

Variation in these *SCCmec* types has made the basis for differentiation among MRSA strains, and each *SCCmec* type encodes for resistance to different antibiotics. *SCCmec* types I (34.3 kb), IV (20.9–24.3 kb) and V (28 kb) encode exclusively for resistance to β-lactam antibiotics [63]. *SCCmec* types II (53.0 kb) and III (66.9 kb) determine multiresistance, as these cassettes contain drug resistance genes on integrated plasmids: pUB110, pI258, pT18 and a transposon Tn554 that confers additional resistance to kanamycin, tobramycin, bleomycin, heavy metals, tetracycline, lincosamide and streptogramin [23, 62].

The *mec* complex also contains the insertion element IS431*mec*, which has been frequently associated with genes encoding resistance to various antibiotics and mercury; in some isolations is also the IS1272 [24]. When regulatory genes *mec*RI (on *SCCmec* types I, IV and V) or *mec*RI and *mec*I (on *SCCmec* types II and III) are intact and fully functional, they appear to confer greater repression on the expression of PBP2a [21, 22, 64, 65].

It has been reported that the *SCCmec* is not restricted to the mobility of the *mec*A gene; he has additional elements, called non-mec, that contribute to the survival and pathogenic potential of *S. aureus*. Among the non-mechanical elements are sequences coding for resistance to heavy metals such as mercury (*SCCmer*) or fusidic acid (SCC MSSA 476, Staphylococcal cassette chromosome methicillin-susceptible *S. aureus*) sequences for biosynthesis capsular (SCCcap1), for the protection of DNA by modification-restriction systems (SCC CI) and for the catabolism of arginine (ACME, arginine catabolic mobile element) [23, 24].

J regions from different *SCCmec* elements are unique to particular types of ccr-mec gene complex combinations and variations of these regions within the same ccr-mec gene complex combination are specific for *SCCmec* subtypes [66, 67]. In the case of *SCCmec*VII and *SCCmec*IX, ccr gene complex positioned between J3 and J2 regions and the *mec* gene complex between J2 and J1 regions is presented [61].

In addition to the *SCCmec* types, several variants of *SCCmec* have been described. Depending on the structural diversity of *mec*I-mecR1 region, six major classes, A–E, of *mec* complexes have been distinguished [67]: Class A, which contains intact *mec* gene complex; Class B, where mecR1 is truncated by insertion sequence IS1272; Class C1, where *mec*R1 is truncated by insertion sequence IS431 having the same direction as the IS431 downstream of *mec*A; Class C2, where mecR1 is truncated by insertion sequence IS431 having the reverse direction to the IS431 downstream of *mec*A; Class D, where *mec*R1 is partly deleted but there is no IS element downstream of Δ*mec*R1 and has been observed in *S. caprae* only. The sixth complex obtained of genome sequence of the bovine *S. aureus* isolate LGA251 assigned as class E [68].

In relation to the genes of the *ccr* complex are designated *ccr*A1 and *ccr*B1 (in *SCCmec* type I), ccrA2 and ccrB2 (in *SCCmec* types II and IV), ccrA3 and ccrB3 (in *SCCmec* type III), ccrA4 and ccrB4 (in *SCCmec type* IV of MRSA strain HDE288) and ccrC (in *SCCmec* type V) [61, 66].

The method of Oliveira and de Lencastre is the most used and cited, which uses the multiplex PCR method for *SCCmec* types I–IV, to detect six gene loci and the *mec*A gene in the *SCCmec* complex [91]. Zhang et al. used a multiplex PCR for the characterization of *SCCmec* types I–V and differentiate between subtypes of *SCCmec* IV (a–d) [69].

Classification scheme of Chongtrakool et al. for the nomenclature of *SCCmec* is based on the *ccr* genes (indicated by a number) and the mec complex (indicated by an upper-case letter). Application of this nomenclature results in *SCCmec* type 1A (type I), type 2A (type II), type 3A (type III), type 2B (type IV) and type 5C (type V). Differences in the J1 region and the J2–J3 regions are then designated with numbers, for example, *SCCmec* type 2B.2.1 (type IVb). The *ccr* genes and the J regions are numbered in chronological order according to their discovery [70].

A combination of two approaches like *SCCmec* typing along with MLST is recommended for reliable typing for multicentre surveillance, inter-hospital and international transmission and evolution of MRSA strains [71].

Studies have found that healthcare associated MRSA (HA-MRSA) strains contain mainly *SCCmec* type I, type II and type III, while community-associated MRSA (CA-MRSA) strains contain type IV and type V cassettes, although several variants have also been reported [72].

4.9.6. Toxin gene profile typing

Studies have shown that MRSA strains possess more toxin genes as compared to MSSA strains. The pathogenicity of *S. aureus* is determined by a variety of bacterial cell wall surface components and exoproteins including toxic shock syndrome toxin (TSST-1), enterotoxins, exfoliative toxins and Panton Valentine leukocidin (PVL) [73].

The *PVL* genes are predominantly associated with *S. aureus* strains that cause community-acquired infections, including skin and soft-tissue abscesses, necrotizing pneumonia and

invasive osteomielitis [74]. These genes are carried on bacteriophages and are easily transferred between lineages. Thus toxin gene profile of the strains can be used as an important epidemiological marker for typing of MRSA strains [75]. MRSA strains isolated from different geographical areas have shown to possess distinct toxin gene profiles. Studies on toxin gene profile of MRSA have reported that most of the CA-MRSA possess genes for PVL toxins and may have evolved from the established CA-MSSA (community-acquired methicillin sensitive *S. aureus*) strains [76]. Of the various methods available, multiplex PCR technique is recommended for detection of toxins in MRSA. It is rapid, reproducible relatively inexpensive, easier to interpret and provides a high degree of discrimination. The technique is useful for studying the chromosomal diversity and evolutionary history of MRSA strains [75].

Today, a greater discrimination such as provided with whole-genome sequencing (WGS) and single-nucleotide polymorphism (SNP) analysis would be useful. High-resolution phylogenetic and phylogeographic (phylodynamic) analyses based on genome-wide SNP data are a powerful tool to infer the origin and test spatiotemporal hypotheses of MRSA spread [77, 78].

The evolutionary rate of MRSA genome-wide SNPs estimated by Gray et al. demonstrates that bacterial genomes can indeed contain sufficient evolutionary information to elucidate the temporal and spatial dynamics of transmission. In the case of HA-MRSA ST239 strain, phylogeographic analyses statistically supported the role of human movement in the global dissemination of this strain [79].

5. Genetic structure of the population of *S. aureus*

The molecular typing techniques have been used in combination to elucidate and study the population structure of *S. aureus* [80–82].

Accordingly, combinations of DNA band-based techniques with DNA sequence-based techniques are frequently used to differentiate between MRSA strains at the local and the international levels [8, 55, 56].

These techniques confirmed the notion that *S. aureus* is a polymorphic species with a clonal population structure [55, 56, 82] that does not undergo extensive recombination, diversifies largely by nucleotide mutations and shows a high degree of linkage disequilibrium (nonrandom associations between genetic loci).

Molecular evolution of MRSA has been favored by horizontal gene transfer [56] and clonal dissemination of certain strains [83–85].

Although *S. aureus* is considered to be an opportunistic pathogen, it is possible that certain clones are more prone to cause invasive disease than are others, due to the presence of virulence factors that increase their chance of gaining access to normally sterile sites [86, 87].

MLST group strains into sequence types (STs) has been used in conjunction with PCR analysis of *SCCmec* element to define the clonal type of MRSA strains (CCs) [55, 58]. Enright et al. [84] using both methods found five clonal complexes found among the population from Southern

Europe, the USA and South America, and defined as groups of isolates from more than one country with the same ST and *SCCmec* type, belonged to one of five clonal complexes, namely the Iberian (ST 247-MRSA-IA), Brazilian (ST239-MRSA-IIIA), Hungarian (ST239-MRSA-III), New York/Japan (ST5-MRSA-II) and Pediatric (ST5-MRSA-IV) clones.

It was shown that, different *SCCmec* types have been acquired by *S. aureus* strains with different genetic backgrounds, and this suggests that SCCmec was introduced several times into different *S. aureus* genetic lineages. ST8-MSSA in CC8 was shown to be the ancestor of the first MRSA strain isolated, that is, ST250-MRSA-I, with ST250 differing from ST8 by a point mutation in the yqiL gene. ST8-MSSA is a common cause of epidemic MSSA disease, and has acquired *SCCmec* types I, II and IV [88].

Another clone that is related closely to ST250 is ST247-MRSA-I, that is, the Iberian clone. These STs differ from each other by a single point mutation at the *gmk* locus. ST247-MRSA-I is one of the major MRSA clones isolated currently in European hospitals [84], and major ST within CC8 is ST239-MRSAIII, which corresponds to the Brazilian clone [86]. This clone has evolved by the transfer, through homologous recombination, of a 557-kb fragment of the chromosome of ST30 into ST8-MRSA-III.

Furthermore, MLST analyses showed that some of the first vancomycin-intermediate *S. aureus* isolates have emerged from ST5-MRSAII, a pandemic MRSA clone known as the NewYork/Japan clone [87, 88]. It has also been shown that multiple lineages of *S. aureus* harbor different *SCCmec* types among hospitalized patients in Australia [89].

Enright et al. in their study found that MRSA has emerged at least 20 times following acquisition of *SCCmec*, and that the acquisition of *SCCmec* by MSSA was fourfold more common than the replacement of one SCCmec with another. Interestingly, *SCCmec* type IV was found in twice as many MRSA clones as other *SCCmec* types, suggesting that most clones arise by acquisition of *SCCmec* type IV by *S. aureus* [90]. This is probably a result of the smaller size of *SCCmec* type IV compared with other *SCCmec* types, which may facilitate transfer of the cassette among staphylococcal species [98]. Furthermore, it has been shown that MRSA strains that belong to the major CCs (1, 5, 8, 22, 30, 45) are easier to transform with *mec*A-expressing plasmids tan are strains belonging to minor CCs. This indicates that the genetic background of *S. aureus* may be important for the stability of *SCCmec* [4, 91].

The population structure of MSSA is genetically more diverse than that of MRSA, and that MRSA originated from a limited number of epidemic MSSA lineages through transfer of the *SCCmec* [92, 93]. It was shown that CC5, 22, 30 and 45 were all derived from epidemic MSSA lineages that have acquired *SCCmec*, since they differed from each other, and from ST8, at six or seven loci [90]. This suggests that some MSSA genetic backgrounds may not provide a stable genetic environment for *SCCmec* integration.

6. Epidemiology of methicillin-resistant *S. aureus*

MRSA first appeared among hospital isolates of UK in 1961 [94] corresponded to *SCCmec* I and it was a typical representative of the archaic clone that rapidly spread in European

countries [4]. These strains, described as epidemic MRSA (EMRSA or HA-MRSA) spread gradually throughout most hospitals all over the world [83, 84, 90]. In the 1970s, MRSA isolates appeared in the USA, Australia and Japan.

In 1982, MRSA *SCCmec* type II was discovered in Japan, and the new York/Japan clone, to which it belongs, also spread, after which the isolation of the MRSA strain *SCCmec* type III followed, in New Zealand [4, 84, 88]. In Asian countries, two epidemic clones, the Brazilian clone (sequence type 239 [ST239]-MRSA-IIIA) and the New York/Japan clone (ST5-MRSA-II) have been found to be prevalent and to possess unique geographic distributions [95]. In central Europe, a close relative of the well-described ST5 MRSA clone, namely ST225, as prevalent in health care setting [54, 90]. This spread from Europe to the USA [54]. In Africa the presence of the following clones: sequence type (ST) 5-MRSA-I, ST239-MRSA-III, ST612-MRSA-IV, ST36-MRSA-II and ST22-MRSA-IV have been reported [97, 98]. ST239 is also common in mainland Asia, South America and parts of Eastern Europe [54]. In the genomes of 63 globally distributed ST239 isolates, SNPs with highly similar sequences between strains from Portugal and South America, which is suggestive of the historical and modern links between these two regions were identified [91].

The particularity of the population structure of MRSA isolations in Latin America was the predominance of only two clones, the Brazilian clone (CC8-ST239-*SCCmec*III) in the strains from Brazil, Argentina, Chile and Uruguay and the Chilean/Cordovan clone [99, 100].

HA-MRSA is mainly multi-resistant, and the choice of antibiotics for treating infections caused by hospital-acquired MRSA is limited to vancomycin and linezolid and mainly causes serious infections in patients who are predisposed in some way: those with a weak immune system, after long-term hospitalization, long-term use of antibiotics, a progressive underlying illness, etc. infection by MRSA strains in hospital conditions is usually preceded by colonization of differing duration [88].

In the 1990s, a new type of MRSA appeared in the USA causing infections in the community among healthy and younger people who had no history of hospital admission or medical treatment in the previous year was reported in Western Australia [70]. These types of MRSA strains were described as CA-MRSA [85, 93].

HA-MRSA strains are genetically distinct to CA-MRSA [101]. Particularly, CA-MRSA strains are usually sensitive to antibiotics other than β-lactams and contain staphylococcal and carry a smaller version of the genetic region responsible for methicillin resistance (*SCCmec* IV or *SCCmec* V), and often produce the Panton-Valentine leukocidin (PVL) [74, 75].

CA-MRSA strains in the USA are most commonly in a genetic cluster designated as PFGE type USA300, MLST type ST8 or spa type t008 [93]. The clonal complexes determined in the SARM-AC strains correspond to CC1 (ST1-SARM-IV) circulating in Asia, Europe and USA, the CC30 (ST30-SARM-IV), CC8 (ST239-SARM-III/IV) detected in Australia, Europe and South America and the USA300 (ST8-SARMI-IV) with a wide geographic distribution which includes countries in Europe and Latin America and in the USA. Also the ST59 in Asia and the USA and the ST80 in Asia, Europe and the Middle East [84, 95, 96, 100]. A variant of clone CC30 (EMRSA-16/ST36-MRSA-II) that is prevalent in the UK and the clone CC5 (ST125-SARM-IV) circulating specifically in Spain exists [50]. Throughout Europe, the CA-MRSA strain is CC80:ST80-IV is the most predominant [83, 84].

The information gathered from MLST indicates that MRSA has evolved multiple times, leading to the circulation and predominance of particular clonal complexes and sequence types [55]. In the case of *SCCmec* type IV, CA-MRSA is an element smaller than the other elements, appears more genetically mobile and does not, at present, carrying additional antimicrobial resistance genes is presented [70]. It also appears to occur in a more diverse range of MSSA genetic backgrounds, suggesting that it has been heterologously transferred more readily from other staphylococcal species [54, 101, 102].

Oosthuysen et al. found a high PVL prevalence, especially among MSSA clones [98]. The MSSA population identified and studied could act as a potential reservoir for CA-MRSA clones upon the acquisition of *SCCmec* elements, leading to the rise of PVL-positive CA-MRSA clones [75, 98].

With the studies of molecular typing in *S. aureus*, they have managed to establish the structural differences between the bacteria isolates and the dynamics of dissemination and the characteristics of the isolates in an outbreak.

Molecular epidemiology studies in MRSA show the predominance of number small clones around the world, that is, they have a capacity for dissemination pandemic, probably favored by cross infections with strains closely related between hospitals from faraway places.

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