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Whole-Genome Molecular Characterization and Typing of *Staphylococcus aureus* Isolated from Lebanon

By

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Whole-Genome Molecular Characterization and Typing of *Staphylococcus aureus* Isolated from Lebanon

Sarah El Hassan

Abstract

Staphylococcus aureus is one of the major causes of nosocomial and community-acquired infections. The rising prevalence of multidrug-resistant (MDR) *S. aureus* in both community and hospital settings is a significant public health concern. In this study, whole-genome sequencing was used for the molecular characterization of 31 *S. aureus* clinical isolates. Genetic relatedness of the isolates was studied using different typing approaches, including Multilocus Sequence Typing (MLST), *S. aureus* protein A (*spa)* typing, and Staphylococcal Chromosomal Cassette (SCC*mec*) typing of the Methicillin-Resistant *S. aureus* (MRSA) isolates. The antimicrobial susceptibility patterns were determined, and virulence determinants were detected *in silico*. Most of the studied isolates carried the *blaZ* gene*,* 45% were MRSA, and 32% were MDR. Fifteen distinct MLST allelic profiles were identified with the most common STs being ST80 (22.58%), ST6 (12.9%), and ST1 (12.9%). Nineteen different *spa* types were detected, with the major ones being t044 (19.35%), t127 (12.9%), and t304 (9.67%). SCC*mec* typing of MRSA revealed the prevalence the type IV, and the most common strain was MRSA ST80-t044-IV. The worldwide dissemination of the MRSA-ST80 clone decreased over the past few years, but it was still the most common strain in Lebanon. For the first time, we detected an untypeable SCC*mec* cassette harboring multiple *ccr* complexes or missing some of the conserved genes. Using the SCCmecFinder, we were able to predict the *mec* cassettes and

showed that the SSA11 harbored a pseudo-SCC*mec* element resembling the one detected SCC*mec* type IV(2B). This study provided a high-resolution view of the molecular characteristics of *S. aureus* clinical isolates. It confirmed that diverse isolates of MSSA and MRSA are circulating in the country differing in antimicrobial susceptibility and virulence determinants. Our results should be further investigated in multiple-center settings to address the high incidence and burden of *S. aureus* infections in Lebanon.

Keywords: *Staphylococcus aureus,* WGS, MLST, SCC*mec, spa,* Lebanon.

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LIST OF ABBREVIATIONS

agr: Accessory gene regulator

AME: Aminoglycosides-modifying enzymes

CA-MRSA: Community associated methicillin-resistant *S. aureus*

CHEF: Contour-clamped homogenous electric field

ClfA/B: Clumping factor A/B

DLVs: Double-locus variants

ECM: Extracellular matrix

eDNA: Extracellular DNA

ET: Exfoliative toxins

FIGE: Field inversion gel electrophoresis

FNBP: Fibronectin binding proteins

FNBP: Fibronectin binding proteins

HA-MRSA: Health-care associated methicillin-resistant *S. aureus*

HAIs: Health-care associated infections

IS: Insertion sequence

MDR: Multi-drug resistant

MGE: Mobile genetic element

MLST: Multi-locus sequence typing

MRSA: Methicillin-resistant *S. aureus*

MSCRAMM: Microbial surface components recognizing adhesive matrix molecules

MSSA: Methicillin-susceptible *S. aureus*

PBP 2a: Penicillin binding protein 2a

PFGE: Pulsed-field gel electrophoresis

PFT: Pore forming toxin

PVL: Panton-Valentine Leukocidin

RFLPs: Restriction fragment length polymorphisms

S. aureus: Staphylococcus aureus

SAB: *S. aureus* bacteremia

Sags: Superantigens

SCC*mec:* Staphylococcal cassette chromosome *mec*

SE: Staphylococcal enterotoxins

SLVs: Single-locus variants

spa-CCs: *spa*-complexes

spa: Staphylococcal protein A

SSSS: Staphylococcal scalded skin syndrome

ST: Sequence type

Tn: Transposons

TSS: Toxic shock syndrome

TSST-1: Toxic shock syndrome toxin 1

VFs: Virulence factors

VISA: Vancomycin intermediate *S. aureus*

VNTR: Variable number tandem repeats

VRSA: Vancomycin resistant *S. aureus*

WHO: World Health Organization

Chapter One

Introduction

1.1. Overview of Staphylococcus aureus

Sir Alexander Ogston, first introduced the name “staphylococcus” during the 1880s when he showed that multiple human pyogenic diseases were correlated with cluster forming microorganisms (Greenwood D., 2012). *Staphylococcus* is now used as the genus name for bacteria that are gram-positive, non-motile, non-spore forming and facultatively anaerobic (Greenwood D., 2012; Namvar et al., 2014). This group is further classified into two main groups, coagulase-positive and coagulase-negative (Namvar et al., 2014).

Staphylococcus aureus falls under the coagulase-positive group and it colonizes the nasal cavity of 20–30% of the human population without causing any apparent disease (Plata, Rosato and Wegrzyn, 2009). The term ‘aureus’ refers to the yellow colonies formed by the bacterium when grown on a nutrient rich agar medium (Gnanamani, Hariharan and Paul-Satyaseela, 2017). The bacteria can either be a commensal bacterium, as it can be found asymptomatically inhabiting the nares, skin, or gastrointestinal tract of humans, or it can act as a pathogen under opportunistic conditions (Greenwood D., 2012; Plata et al., 2009).

S. aureus infection results in a large burden of diseases within both hospital environments and community settings (Feil et al., 2003). There is an increased risk of developing *S. aureus* infections for carriers. The diseases that the bacterium can

manifest range in severity from soft tissue infections to more deep-seated infections such as septicemia, endocarditis, bacteremia and osteomyelitis (Jenkins et al., 2015). This bacterium has been the leading cause of health-care-associated infections (HAIs) for years and it plays a role in 16–21% of infections in both the developed and developing world (Greenwood D., 2012). *S. aureus* is the second most frequently reported causative agent of health care associated infections (HAIs) requiring antibiotic treatment in US hospitals. The organism is increasingly showing resistance to multiple antimicrobial agents (Greenwood D., 2012). *S. aureus* bacteremia (SAB) was linked to high mortality rates reaching up to 30% (van Hal et al., 2012), with the World Health Organization (WHO) classifying *S. aureus* among the high priority antibiotic-resistant pathogens that pose a risk to human health (Who.int., 2020).

The emergence and spread of Methicillin-resistant *Staphylococcus aureus* (MRSA) was implicated as one of the major events in the epidemiology of infectious diseases (Lee et al., 2018).

1.2. Epidemiology of *S. aureus*

After penicillin's widespread introduction during 1944, the world saw a swift emergence of *S. aureus* strains that are resistant to the antibiotic, with the penicillinase producing *S. aureus* hospital isolates raising to up to 50% by 1948 (Livermore, 2000). Resistance to penicillin continued to increase in subsequent years leading to the development of other antibiotics (Livermore, 2000). Methicillin, a semi-synthetic penicillin, was introduced as a drug option to combat penicillin resistant *S. aureus* in 1961. Unfortunately, resistant strains were reported soon after the introduction of methicillin into clinics (Lee et al., 2018). This resistance was the outcome of the incorporation of the *mecA* or *mecC* genes, carried on the staphylococcal cassette chromosome *mec* (SCC*mec)* element (Lakhundi and Zhang, 2018). The following

decade was marked with outbreaks caused by methicillin resistant *S. aureus* (MRSA), especially in European countries (Lee et al., 2018). Initially these outbreaks were health-care associated MRSA (HA-MRSA), but a change in MRSA's epidemiology was seen when a new MRSA classification, community-associated MRSA (CA-MRSA), was presented (Lee et al., 2018). CA-MRSA strains were detected when MRSA strains from individuals who have not had any contact with hospital facilities were being reported in the 1980s (Dukic et al., 2013). One notable feature of CA-MRSA strains is that they have very active Agr systems compared to HA-MRSA and that allows them to release more toxins and have increased virulence (Lee et al., 2018). The classification of MRSA isolates as either HA or CA has become increasingly more difficult, and that is because both CA-MRSA and HA-MRSA can now be found circulating in both community and hospital settings (Kateete et al., 2019).

Currently, MRSA is widespread and appears to be endemic in most hospitals in Asian countries, with up to 70% of *S. aureus* clinical isolates being resistant to methicillin in South Korea (Lee et al., 2018). Borg et al. showed that hospitals located in multiple south-eastern Mediterranean countries had hyperendemicity in MRSA strains (Borg et al., 2007).

1.3 Biofilm formation

In order to survive in challenging environments, some bacteria form aggregations known as biofilms. Biofilm formation is essentially divided into three major events (Lister and Horswill, 2014). The first being the initial attachment of the bacterial cell to the surface, which is mediated by bacterial surface proteins (Lister and Horswill, 2014). Maturation of the biofilm is associated with the production of extracellular matrix (ECM) being composed of proteins, polysaccharides, or extracellular DNA (eDNA) (Lister and Horswill, 2014). Biofilm dispersal is linked to

the degradation of the extracellular polymeric matrix (Lister and Horswill, 2014). *S. aureus* is notorious for causing biofilm-related infections formed on indwelling medical devices such as catheters (Moormeier and Bayles, 2017). *S. aureus* is often a commensal bacterium found on the human skin and so infection is likely to happen during invasive procedures such as when a medical device is inserted during a surgery (Al-Mebairik, El-Kersh, Al-Sheikh and Marie, 2016). Cells within a biofilm have higher tolerance to antibiotics and were linked to the progression of chronic diseases (Moormeier and Bayles, 2017).

The quorum-sensing accessory gene regulator (*agr*) system controls multiple virulence factors produced by *S. aureus* and it plays a crucial role in biofilm formation (Yarwood, Bartels, Volper and Greenberg, 2004). The *agr* locus is made up of two distinctive operons driven by the P2 and P3 promoters. The operon driven by P2 consists of *agrBDCA* and it codes for the RNAII transcript whereas the one driven by P3 drives the transcription of the effector molecule of this locus (RNAIII) (Yarwood et al., 2004). Characterization of the *agr* locus revealed that it has four variants (type I-IV) that must have evolved in order to preserve *agr* functionality and aid the bacterium in evading host defenses and spreading within the host (Tan et al., 2018).

1.4. Virulence Factors

Virulence in *S. aureus* and other bacteria is often associated with mobile genetic elements (MGEs), such as bacteriophages, plasmids and pathogenicity islands that mediate the transfer of genetic material (DNA) among bacterial populations (Malachowa and DeLeo, 2010). There are three ways for bacteria to obtain genetic material from other cells in their surrounding environment: (1) uptake of free DNA from the environment (transformation), (2) bacteriophage transduction, and (3) direct cell contact (conjugation) (Malachowa and DeLeo, 2010). *S. aureus* contains many

types of MGEs, including plasmids, transposons (Tn), insertion sequences (IS), bacteriophages, pathogenicity islands, and staphylococcal cassette chromosomes (Malachowa and DeLeo, 2010).

S. aureus expresses a wide range of virulence factors that helps it to survive and spread in the human host once it breaches the primary defense mechanisms. These virulence factors are essentially divided by their mechanism of action (Al-Mebairik et al., 2016). These mechanisms can either be factors that aid the pathogen in adhering and invading host cells or secreted toxins that could cause tissue damage and promote the spread of the bacterium in the host (Al-Mebairik et al., 2016).

1.4.1 Adherence factors

In order to initiate colonization and infection *S. aureus* adheres to the extracellular components of the host through the surface protein adhesins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family (Bien, Sokolova and Bozko, 2011). This major class of proteins allow the bacterium to adhere to the plasma and extracellular matrices of the host such as collagen, fibrinogen and fibronectin (Bien et al., 2011). *S. aureus* expresses either one of the two fibronectin binding proteins (FNBPs), FnBPA or FnBPB (Josse, Laurent and Diot, 2017). FNBPs are virulence determinants linked to host invasion (Josse et al., 2017), and are encoded by tandem genes (*fnb*A and *fnb*B, respectively), that are transcribed separately (Josse et al., 2017). The role of FNBPs goes beyond mediating the interaction of *S. aureus* with fibronectin; intracellular invasion of the bacterium is dependent on FNBPs (Josse et al., 2017). A model for the process of internalization was proposed. Fibronectin acts as a bridge between the FNBPs and the host integrin receptor a5b1 (Josse et al., 2017).

Other MSCRAMM proteins include the clumping factor A (ClfA) and ClfB, which are the fibrinogen binding proteins that can cause platelet aggregation in the host's blood plasma (O´Brien et al., 2002).

1.3.2 Secreted factors

Other than the cell surface factors, *S. aureus* is also able to produce a barrage of extracellular toxins. These include the several types of staphylococcal enterotoxins (SEs), which are mainly responsible for staphylococcal food poisoning (Bien et al., 2011). Other types of secreted staphylococcal toxins include toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETs), hemolysis (alpha, beta, gamma and delta) and Panton-Valentine Leukocidin (PVL) (Bien et al., 2011). TSST-1 is an exotoxin belonging to the superantigens (SAgs) family of toxins along with the SEs (Xu and McCormick, 2012). TSST-1 is deemed to be the major cause of the menstrual form of toxic shock syndrome (TSS), particularly in women that use tampons (Xu and McCormick, 2012).

On the other hand, the α-hemolysin (α-toxin) encoded by *hla* is a pore-forming toxin (PFT) and is the most well-characterized. PVL, along with γ-hemolysin, belong to the family of synergohymenotropic toxins which cause membrane damage and lysis of host defense cells such as monocytes and macrophages (Lina et al., 1999). The toxin is made of two proteins S and F that are designated as LukS-PV and LukF- PV, respectively (Lina et al., 1999). The two subunits are encoded by *LukS-PV* and *LukF-PV*, carried on bacteriophages (i.e., ΦSa2958, ΦPVL and ΦSa2USA) (Boakes et al., 2010). *S. aureus* was also found to produce exfoliative toxins (ETs), namely serine proteases and which are linked to the pathogenesis of staphylococcal scalded skin syndrome (SSSS) (Bien et al., 2011).

1.5 Antimicrobial resistance

1.5.1 Penicillin and Methicillin resistance

The introduction of antimicrobial agents for treatment of infectious diseases is one of the major achievements of the 20th century (Zankari et al., 2012). Unfortunately, isolates with acquired resistance emerged for some of these antibiotics soon after their introduction and this pattern has followed with the introduction of each new antimicrobial agent. *S. aureus* is naturally susceptible to most antibiotics but the bacterium is increasingly showing resistance to multiple antimicrobial agents (Zankari et al., 2012). The continuous evolution of antimicrobial-resistance emanates from multiple reasons such as: the widespread and sometimes misuse of antimicrobials, the excessive use as growth enhancers in livestock animals, and the widespread distribution of resistant bacteria linked to the increase in regional and international travel (Lowy, 2003).

Penicillin-resistant staphylococci were first detected in hospitals during 1942, resistance to penicillin is linked to β-lactamase which is encoded by the *bla*Z gene; an enzyme that hydrolyzes the β-lactam ring inactivating the drug. *blaZ* is carried on a plasmid and it is under the control of two adjacent regulatory genes, the anti-repressor *blaR1* and the repressor *blaI* (Lowy, 2003).

Outbreaks linked to MRSA were reported across Europe and the USA following its first detection in the United Kingdom (Peacock and Paterson, 2015). Resistance to methicillin is intrinsic and not mediated by a plasmid-borne β-lactamase (Peacock and Paterson, 2015). *mecA* gene encodes for the modified penicillin binding protein 2a (PBP 2a or PBP 2') which leads to reduced affinity for β-lactams (Al-Mebairik et al., 2016). The mobile genetic element (MGE) carrying *mecA* is the staphylococcal chromosome cassette (SCC*mec*) which is present in MRSA but absent

from methicillin-susceptible *S. aureus* (MSSA). The SCC*mec* element is a 21 to 60 kb in size being diverse due to the presence of multiple types and subtypes (Wielders et al., 2002). The element contains three essential genetic components; the *mec* gene complex which consists of the methicillin resistance gene *mec* (*mecA, mecB, mecC and/ or mecD*) and its control elements (*mecR* and *mecI*); the *ccr* gene complex that carries the site-specific recombinase genes *ccrA* and *ccrB,* which allow the movement of the SCC*mec* element; and the J regions (Lakhundi and Zhang, 2018). The SCC*mec* element integrates into the *S. aureus* genome at an *attB* integration site sequence present at the 3′ end of the *orfX* gene (Peacock and Paterson, 2015). SCC*mec* classification approach is also used for the typing of *S. aureus*.

1.5.2 Vancomycin resistance

Vancomycin became the drug of choice for the treatment of MRSA infections during the 1980s (ElSayed, Ashour and Amine, 2018). *S. aureus* strains with reduced susceptibility to vancomycin were first identified in 1996, when a strain (Mu 50) with a vancomycin MIC of 8 mg/L was isolated in Japan. From then on, more cases of vancomycin intermediate (VISA) or resistant *S. aureus* (VRSA) began to emerge throughout the world especially in developing countries. The first *S. aureus* isolates exhibiting high-level of vancomcyin resistance (MIC ≥16 g/ml) were isolated in 2002 in Michigan and Pennsylvania and in 2004 in New York (Chen and Huang, 2014). The strains carried plasmid-borne copies of the transposon Tn*1546,* which was acquired from vancomycin-resistant *Enterococcus faecalis* (Gardete and Tomasz, 2014). Transposon Tn1546 carries the *vanA o*peron (consisting of *vanA*, *vanH*, *vanX*, *vanS*, *vanR*, *vanY* and *vanZ*) which reduces the susceptibility of *S. aureus* to vancomycin (McGuinness et al., 2017). Chamoun et al. (2016) revealed that out of 4890 *S. aureus* strains collected throughout 2011-2013 from 16 different Lebanese hospitals, none

was VISA or VRSA (Chamoun et al., 2020).

1.5.3 Quinolones resistance

Quinolone resistance among *S. aureus* emerged most notably among the methicillin-resistant strains. As a result, the ability to use fluoroquinolones as anti-staphylococcal agents was considerably reduced. Quinolones act on DNA gyrase and topoisomerase IV and inhibit their activity of separating concatenated DNA strands (Lowy F., 2003). Three mechanisms concerned in fluoroquinolone resistance are the following: topoisomerase IV gene mutations, DNA gyrase gene mutations and expression of the NorA efflux pump. DNA topoisomerase IV is composed of the GrlA and GrlB subunits, encoded by the *grlA* and *grlB* genes respectively, mutations in either one of these genes can lead to quinolone resistance (Wang T. et al., 1998).

1.5.4 Resistance to other antibiotics

HA-MRSA strains are usually found to be multidrug resistant (MDR) bacteria, with resistance to tetracyclines, aminoglycosides and multiple other drugs often reported (Gajdács, 2019). Many resistance mechanisms found in *S. aureus* strains are due to the acquisition of mobile genetic elements. Two mechanisms of resistance to tetracyclines are known in *Staphylococcus*: active efflux pumps encoded by plasmid-located genes, *tetK* and *tetL* and ribosomal protection mediated *TetM* determinants that are carried on the Tn*916*-like transposon Tn*5801* (Jensen and Lyon, 2009). *S. aureus* aminoglycoside resistance is through the cytoplasmic aminoglycoside modifying enzymes encoded by varying transposons (Foster, 2017).

1.6 Typing

With the frequency at which MRSA infections are being recorded, especially in the intensive care setting, calls for the need to have reliable and efficient methods

to characterize isolates and have proper identification of the clonal spreading within a certain region and in hospitals (Lakhundi and Zhang, 2018). Knowledge on the dissemination of the organism will enable the implementation of effective control measures that will limit or seize its spread (Lakhundi and Zhang, 2018). Multiple DNA-based typing methods are used and applied by clinicians and epidemiologists to characterize isolates in order to distinguish between related and unrelated strains. This ability to identify strains allows in determining whether the patient is having successive or recurrent infections, it also gives insight into the possibility of an outbreak in a particular setting (Lakhundi and Zhang, 2018).

When evaluating typing techniques, two general criteria are looked into, which are performance/efficacy and convenience/efficiency (Lakhundi and Zhang, 2018). Typeability, reproducibility and discrimination ability fall under the performance/efficacy. Rapidity, affordability and technical simplicity however, fall under convenience/efficiency (Lakhundi and Zhang, 2018). Due to the fact that MRSA are derived from a single clone or at least few precursor strains, the methods used to distinguish MRSA from each other should be discriminatory (Schmitz et al., 1998).

Multiple techniques have been developed over the last decades and each of these has its own limitations and advantages. There are currently four widely used typing methods of *S. aureus* including pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), *spa* typing and SCC*mec* typing (Lakhundi and Zhang, 2018).

1.6.1 Pulsed-Field Gel Electrophoresis

Analysis of DNA fragments using PFGE, was first described by Shwarz and Cantor and was considered as the golden standard for MRSA typing (Schwartz and Cantor, 1984). This approach entails the usage of restriction endonucleases that

recognize specific sites on the bacterial genome and digest producing different sized fragments. To achieve size separation, alternating voltage gradient is used (Lakhundi and Zhang, 2018). The DNA should remain intact and so bacterial cells are contained within low-melting-point agarose plugs (Goering 2010). Then DNA molecules within the agarose plugs are subjected to restriction digestion using in the case of *S. aureus smaI* (5'-CCC^GGG-3') that cuts A+T rich regions in Gram-positive bacteria (Goering 2010). *smaI* was found to be ideal for MRSA typing out of multiple available restriction endonucleases giving reproducible and accurate results (Lakhundi and Zhang, 2018). Following restriction digestion, the plugs are loaded on agarose gel. Switching the direction of the electrical current in a prearranged pattern allows for large DNA fragments to separate since reorientation and migration are size dependent. There are two approaches for fragment separation in PFGE: field inversion gel electrophoresis (FIGE) and contour-clamped homogenous electric field (CHEF) (Lakhundi and Zhang, 2018). FIGE involves the reorientation of the angle of the electric field and is best used to separate fragments that range between 0.1 and 200 kb. CHEF on the other hand, generates uniform electric field and is best suited for the separation of fragments that are up to 3 Mb and it is the most commonly used approach (Lakhundi and Zhang, 2018).

PFGE is one of the most commonly used typing approach, and so multiple protocols and data analysis tools were developed (Neoh, Tan, Sapri and Tan, 2019). PFGE output is seen as a banding pattern distinguishing between closely related isolates assorting them into distinct pulsotypes, if the difference is in three bands and more (Neoh et al., 2019; Tenover et al., 1995).

PFGE however has some limitations including that it is time-consuming and labor intensive, and cannot be used to compare results between labs (Lakhundi and

Zhang, 2018).

1.6.2 Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) is another typing approach used for the characterization of *S. aureus*. First introduced in 1998, MLST is a genotyping method that entails the sequencing of seven constitutively expressed housekeeping genes (Ji, 2020). A 450-500 bp fragment is obtained for each locus after PCR amplification and is then assigned to an allele number based on the detected sequence (Lakhundi and Zhang, 2018). The sequence type (ST) is based on the allelic assignment for each of the seven housekeeping genes (Liu and Ji, 2020).

MLST has been used and validated since 2000 as a genotyping method for *S. aureus,* including MSSA and MRSA isolates (Saunders and Holmes, 2007). The seven *S. aureus* housekeeping genes range in size between 402-516bp and code for carbamate kinase (*arcC)*, shikimate dehydrogenase (*aroE)*, glycerol kinase (*glpF)*, guanylate kinase (*gmk)*, phosphate acetyltransferase (*pta)*, triosephosphate isomerase (*tpi)* and acetyl coenzyme A acetyltransferase (*yqiL*) (Lakhundi and Zhang, 2018). The combination between the different alleles determines the sequence type (ST) using freely available databases such as the MLST website (http://www.mlst.net) (Lakhundi and Zhang, 2018). The MLST scheme for *S. aureus* can be found on http://www.saureus.mlst.net/, with new alleles and STs being constantly updated. MLST was used to study the evolution of *S. aureus* strains and the worldwide dissemination of certain clones (Lakhundi and Zhang, 2018).

Clusters of closely related genotypes dominate and are known as clonal complexes, with each clonal complex consisting of strains that have at least 5 out of the 7 common alleles (Feil et al., 2003). Strains that have the same ST are from the

same genetic lineage and those that have a one or two loci difference are considered as single-locus variants (SLVs) and double-locus variants (DLVs), respectively (Lakhundi and Zhang, 2018). Additionally, an algorithm, known as eBURST, was also developed that can be used to determine the evolutionary a (Lakhundi and Zhang, 2018).

1.6.3 *spa* typing

The *spa* gene codes for staphylococcal protein A, which is a conserved cell-wall protein in *S. aureus*. *spa* typing was adapted as another characterization approach that has a discriminatory power falling between PFGE and MLST (Lakhundi and Zhang, 2018). The gene is around 2,150 bp and encodes for the following regions: Fc-binding region, the X region and the C-terminal region (Tang et al., 2000). The X region of the protein A gene consists of variable number tandem repeats (VNTRs) (Tang et al., 2000). These VNTRs comprise 2-15 repetitive sequences that display extensive polymorphism based on point mutations, deletions, duplications, and insertions (Lakhundi and Zhang, 2018). The *spa* type is determined by the pattern of the repeat units, meaning two isolates that have the same sequence and order of the repeat units are genetically related (Lakhundi and Zhang, 2018). The *spa* typing method is cost effective, reproducible and easy to interpret since it involves analyzing one single polymorphic locus (Lakhundi and Zhang, 2018), making it ideal for the investigation of outbreaks and local epidemiological studies.

MRSA *spa*-based clustering appeared to be stable over time and so an algorithm, BURP, was introduced to cluster MRSA isolates into *spa*-complexes (*spa*-CCs) (Mellmann et al., 2008). The *spa* types and *spa* repeat sequences are all available on a central *spa* server at www.spaServer.ridom.de/. Submission for new *spa* types

can be done through the server which would be added to the reference database. This accessibility to a common database as well as the reproducibility and rapidity made *spa* typing a frequently used method for characterization of *S. aureus* isolates. The downside to this method however, is the typing being based on a single-locus, which could cause potential misclassifications due to recombination and or sequencing errors (Lakhundi and Zhang, 2018).

1.6.4 SCC*mec* typing

SCC*mec* is a mobile element that is a determinant for ß-lactam resistance as it carries the methicillin resistance gene, *mecA*. To date, there are 13 distinctive types of SCC*mec* detected, and based on the difference in their joining regions they were further divided into subtypes (Lakhundi and Zhang, 2018). Determining the SCC*mec* type became essential in characterizing MRSA isolates. Since 2002, multiple strategies have been suggested for the best *SCCmec* typing scheme and they included restriction fragment length polymorphisms (RFLPs) analysis of PCR or multiplex PCR products and multiplex real-time PCR (Yang, Park, Sohn and Kim, 2006). So far, the most commonly used method is the multiplex PCR developed by Zhang et al. which can characterize SCC*mec* elements types I to V (Zhang et al., 2005). To date we do not have a single PCR assay that can be used to identify all SCC*mec* types and subtypes (Lakhundi and Zhang, 2018). Finally, it is noteworthy that in naming MRSA clones we use both the MLST and SCC*mec* type, such as ST80-IV (Lakhundi and Zhang, 2018).

Objectives

In this study, we aimed at conducting a detailed characterization of 31 *S. aureus* clinical isolates recovered during 2018 from a Lebanese hospital. With a whole-

genome sequencing approach, we determined the genetic characteristics of *S. aureus* and reconstructed the phylogenies of the strains found in the hospital, screened for a panel of known antimicrobial resistance genes and virulence genes, and tested for the presence of an outbreak.

Chapter Two

Materials and Methods

2.1 Bacterial isolate collection

A total of 31 non-duplicate *Staphylococcus aureus* isolates collected throughout 2018 were collected from the Middle East Institute of Health University Hospital (MEIH) and were designated as SSA1 – SSA31. Table 1 summarizes the source of isolation. All the isolates were confirmed as *S. aureus* by further growing them on mannitol salt agar (MSA).

Table 1: Site of isolation for all S. aureus undertaken in this study.

Specimen source	# Found in isolates (%)
Wound/pustule/abscess/carbuncle	18 (58.06%)
Nose/Nose abscess	5 (16.12%)
Urine	2 (6.45%)
Sputum	3 (9.67%)
Blood	1 (3.22%)
Others	2 (6.45%)

2.2 DNA extraction

All isolates were cultured on tryptic soy broth (TSB) for 24h and DNA was extracted using the Nucleospin® Tissue Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Genomic DNA was stored at -20 ^{O}C for downstream processing.

2.3 Antibiotic susceptibility

Antimicrobial phenotypic testing was done through the Kirby-Bauer disk diffusion assay, wherein the turbidity of bacterial-saline suspensions was adjusted to match that of 0.5 Mcfarland standard and then plated on Mueller-Hinton agar for 24h at 37 °C. The isolates were tested against 11 different antibiotics using representatives of 8 different classes. These antibiotics included penicillin, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, cefoxitin, rifampin, tobramycin and tetracycline. The applied breakpoints for fusidic acid (≤1 µg/ml) were ≥22 mm for susceptibility and ≤19 mm for resistance, according to Jones et al. (2010). For the other antimicrobial agents (Table 2), it was determined following the National Committee for Clinical Laboratory Standards Guidelines (CLSI, 2018).

Table 2: Antibiotic zone of inhibition (mm) according to the standards set by CLSI (2018).

	Zone of inhibition (mm)		
Antibiotics	**S**	**I**	**R**
Penicllin (10 units)	≥ 29	-	≤ 28
Cefoxitin (30 µg)	≥ 22	-	≤ 21
Kanamycin (30 µg)	≥ 18	14-17	≤ 13
Tobramycin (10 µg)	≥ 15	13-14	≤ 12
Gentamicin (10 µg)	≥ 15	13-14	≤ 12
Erythromycin (15 µg)	≥ 23	14-22	≤ 13
Clindamycin (2 µg)	≥ 21	15-20	≤ 14
Tetracylcine (30 µg)	≥ 19	15-18	≤ 14
Ciprofloxacin (5 µg)	≥ 21	16-20	≤ 15
Rifampin (5 µg)	≥ 20	17-19	≤ 16

2.4 Biofilm formation Assay

Microtiter-plate assay was performed in order to qualitatively assess the *S. aureus* isolates' biofilm production ability. The assay for biofilm formation was done according to Stepanovic et al. (2007). Briefly, *S. aureus* strains were grown in 5 mL Tryptic Soy Broth (TSB) for 18 h±30 mins at 37 ^{o}C. Following which, 50 µL from each culture was transferred into 5 mL of TSB with 1% glucose to obtain a 1:100 dilution. Aliquots (200 µL) of these suspensions were then distributed into 96-wells plate; three wells/ isolate and six wells for the negative control (broth medium only). The plate was then incubated at 37 ^{o}C for 24h. Following incubation, the wells were decanted and carefully washed three times using 300 µL phosphate-buffered saline (PBS) to remove the non-adherent cells. Biofilms were then fixed with 150 µL 2% crystal violet for 15 min after being heat fixed at 60 ^{O}C for 60 min. Excess dye was removed by washing. After air-drying, 150 µL of 95% ethanol was added to the wells. The OD of the wells was measured at 595 nm using microtiter-plate reader (ThermoScientific, Waltham, MA, USA). The results were evaluated using a scheme defining the cut-off (ODc) value as the average OD of the negative control + three standard deviations of negative control OD. The degree of biofilm formation was scored following the matrix in Table 3.

Table 3: Biofilm scoring matrix

No biofilm production (0)	**OD ≤ ODc**
Weak biofilm production (1)	ODc < OD ≤ 2XODc
Moderate biofilm production (2)	2 ODc < OD ≤ 4X ODc
Strong biofilm production (3)	OD > 4X ODc

2.5 Multiplex PCR Assay for Staph 16S rRNA, *mecA* and PVL Detection

PCR was performed on all *S. aureus* isolates targeting the *lukS*-PV/*lukF*-PV, *mecA* and 16S rRNA to detect the *mecA* and PVL genes. The PCRs were performed using the following primers Staph756F and Staph750R for the 16S rRNA amplification, Luk-PV-1 forward and Luk-PV-2 reverse were used for the detection of *lukS*-PV/*lukF*-PV genes, and primers MecA1 forward and MecA2 reverse for the detection of the *mecA* gene (Table 4) (McClure et al., 2006). The PCR amplification was done using 4 ng/uL DNA in a total volume of 25 µL using 0.2 mM dNTPs, 1.6x Taq buffer, 1.5 mM $MgCl_2$, 0.2 µM of the Staph750F primer, 0.2 µM of the Staph 750R primer, 0.4 mM F Luk-PV-1 primer, 0.4 mM R Luk-PV-2 primer, 0.4 mM F MecA1 primer, 0.4 mM R MecA2 and 2 U DreamTaq polymerase (ThermoScientific, Waltham, MA, USA). The DNA fragments were resolved in a 1.5% agarose gel with 1X Tris-Acetic Acid-EDTA (TAE) buffer ((Bio-Rad, Hercules, CA) at 80 V for 30 mins and were visualized with ethidium bromide. The sizes of the PCR products and the primer sequences are found in Table 4.

Table 4: Primers for Staph 16S rRNA, *mecA* and PVL PCR assay and amplicon sizes.

Primer	Orientation	Sequence (5'-3')	Amplicon Size (bp)
Staph756	F	AACTCTGTTATTAGGGAAGAACA	756
	R	CCACCTTCCTCCGGTTTGTCACC	
mecA	F	GTAGAAATGACTGAACGTCCGATAA	300
	R	CCAATTCCACATTGTTTCGGTCTAA	
LUK-PV	F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433
	R	GCATCAAGTGTATTGGATAGCAAAAGC	

2.6 SCC*mec* Typing

SCC*mec* was done using three multiplex PCR (M-PCR) assays. The 1st M-PCR had nine pairs of primers (Table 5) for the *SCCmec* types and subtypes (I, II, III, IVa, IVb, IVc, IVd and V), the 2nd M-PCR contained four primers for the characterization of the *mecA* complex (mecI-F, mecI-R, IS1272-F and mecR1-R, the 3rd M-PCR also contained four primers for the characterization of the *ccr* gene complex (ccrAB-β2, ccrAB-α2, ccrAB-α3, and ccrAB-α4). For multiplex PCR, the conditions used were as previously described by Zhang et al. (2005). Reaction mixtures for the multiplex PCR assays contained 4 ng/uL of DNA, 50 mM KCL, 2.5mM $MgCl_2$, 0.2mM of dNTPs and various concentrations of the respective primers (Table 5). The PCR products were resolved in a 1.8% (w/v) agarose gel in 0.5% Tris-borate-EDTA buffer (Bio-Rad, Hercules, CA) at 80 V/cm for 30 mins and were visualized with ethidium bromide. Table 5 summarizes the PCR primers used in the study (Zhang et al., 2005).

Table 5: Primers used for SCC*mec* typing (Zhang et al., 2005)

Primers	Orientation	Oligonucleotide sequence (5'-3')	Concentration (mM)	Amplicon size (bp)
Type I	F	GCTTTAAAGAGTGTCGTTACAGG	0.048	613
	R	GTTCTCTCATAGTATGACGTCC		
Type II	F	CGTTGAAGATGATGAAGCG	0.032	398
	R	CGAAATCAATGGTTAATGGACC		
Type III	F	CCATATTGTGTACGATGCG	0.04	280
	R	CCTTAGTTGTCGTAACAGATCG		
Type IV-a	F	GCCTTATTCGAAGAAACCG	0.104	776
	R	CTACTCTTCTGAAAAGCGTCG		
Type IV-b	F	TCTGGAATTACTTCAGCTGC	0.092	493
	R	AAACAATATTGCTCTCCCTC		
Type IV-c	F	ACAATATTTGTATTATCGGAGAGC	0.078	200
	R	TTGGTATGAGGTATTGCTGG		
Type IV-d	F	CTCAAAATACGGACCCCAATACA	0.28	881
	R	TGCTCCAGTAATTGCTAAAG		
Type V	F	GAACATTGTTACTTAAATGAGCG	0.06	325
	R	TGAAAGTTGTACCCTTGACACC		
MecA147	F	GTGAAGATATACCAAGTGATT	0.046	147
	R	ATGCGCTATAGATTGAAAGGAT		
mecI	F	CCCTTTTTATACAATCTCGTT	0.08	146
	R	ATATCATCTGCAGAATGGG		
IS1272	F	TATTTTTGGGTTTCACTCGG	0.08	1,305
mecR1	R	CTCCACGTTAATTCCATTAATACC		
ccrAB-β2	F	ATTGCCTTGATAATAGCCITCT	0.08	
ccrAB-α2	R	AACCTATATCATCAATCAGTACGT	0.08	700
ccrAB-α3	F	TAAAGGCATCAATGCACAAACACT	0.08	1,000
ccrAB-α4	R	AGCTCAAAAGCAAGCAATAGAAT	0.08	1,600
ccrC	F	ATGAATTCAAAGAGCATGGC	0.08	336
	R	GATTTAGAATTGTCGTGATTGC	0.08	

2.6 Pulsed-Field gel electrophoresis (PFGE)

PFGE was performed using *SmaI* (ThermoScientific, Waltham, MA, USA) as described previously (Slama et al. 2012). The universal laboratory standard *Salmonella enterica subsp. enterica serovar Braenderup* (ATCC® BAA664TM) was used as a reference strain. Electrophoresis was performed using the Bio-Rad laboratories CHEF DR-III system (Bio-Rad Laboratories, Bio-Rad Laboratories Inc., Hercules, CA, USA) under the conditions set in the Unified PFGE Protocol for Gram Positive Bacteria (https://www.cdc.gov/pulsenet/). Gels were stained with ethidium

bromide. PFGE profiles were analyzed using the BioNumerics software version 7.6.1 (Applied Maths, Belgium). Fingerprints with a difference of three or more bands were assigned as different pulsotypes. Clustering of the pulsotypes was set using the BioNumerics software analysis through dice correlation coefficients with an optimization of 1% and tolerance of 1%.

2.7 Whole genome sequencing and data analysis

2.7.1 Library preparation

The Illumina Nextera XT (Illumina, San Diego, CA, USA) library preparation kit was used to prepare a library using 1 µg of DNA. The Agencourt AMPure XP PCR purification beads (Agencourt, Brea, CA) were used to allow effective clean up after library preparation. Quantitative PCR was performed for the resultant DNA libraries using Qubit. The libraries were sequenced on an Illumina MiSeq with 250 bp x 2 read length.

2.7.2 Quality control and assembly

Genome assembly was performed *de novo* using SPAdes version 3.12.0 (Bankevich et al., 2012). Quality control was done for the raw sequence data using FastQC version 1.0.0. The adapter sequence and low-quality bases were trimmed using Trimmomatic v0.38 (Andrews, 2010; Bolger, Lohse, & Usadel, 2014).

2.7.3 *in silico* resistance, virulence, MLST profiling and detection of mobile genetic elements (MGEs)

For the assignment of gene functions, and the prediction of subsystems in the genome the RAST server was used (http://rast.nmpdr.org) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015). The Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017) and ResFinder 3.0 (Zankari et al., 2012) available on the

Center for Genomic Epidemiology website (www.genomicepidemiology.org) were used to detect resistance genes. The presence of putative virulence factors (VFs) was assessed using VirulenceFinder 1.5 (Joensen et al, 2014). The *agr* types were determined *in silico* using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MLST for each isolate was determined *in silico* by mapping the high throughput sequencing data to the online database on the CGE MLST 1.8 server (Larsen et al., 2012). The presence of plasmids in the genomic sequences was determined using PlasmidFinder 1.3 (Carattoli et al., 2014). The Phage Search Tool Enhanced Release (PHASTER) (http://phaster.ca) was used for the identification of phages (Zhou et al., 2011; Arndt et al., 2016). To further study the diversity of *S. aureus,* genomes were annotated using Prokka (version 1.13) with a similarity cutoff e-value of 10^{-6} and minimum contig size of 200 bp (Seemann, 2014). Annotated GFF3 files were piped into Roary (version 3.12).

Chapter Three

Results

3.1 Antibiotic susceptibility of *Staphylococcus aureus* isolates

All *S. aureus* isolates were tested for their susceptibility against a panel of 11 antibiotics covering eight classes that included penicillin, cephalosporins, aminoglycosides, macrolides/lincosamides, tetracyclines, fluoroquinolones, fusidic acid and rifampicin (Table 6).

Most of the isolates (83.87%; n=26/31) were resistant to penicillin, followed by resistance to fusidic acid and cefoxitin (41.93%; n= 13/31), kanamycin and ciprofloxacin (19.35%; n= 6/31), gentamicin (12.9%; n= 4/31), tobramycin (12.9%; n=4/31), and tetracycline (12.9%; n= 4/31). Ten out of the 31 isolates (32.25%) were multidrug-resistant (MDR) for showing resistance to one or more agents within three or more antimicrobial classes (Magiorakos et al., 2012). All of the MDR strains were MRSA isolates and they were most commonly resistant to fusidic acid (80%; 8/10) and aminoglycosides (60%; 6/10). SSA33 was the only isolate that showed resistance to all 11 of the tested antibiotics, while three of the isolates (SSA18, SSA25 and SSA28) were susceptible to all the tested antimicrobial agents (Table 6).

Table 6: Antibiotic susceptibility testing results against 11 antimicrobial agents representing eight classes including; penicillin, cephalosporins, aminoglycosides, macrolides/lincosamides, tetracyclines, fluoroquinolones, fusidic acid and rifampicin

				Susceptibility Profile										
Isolate ID	Date of Isolation	Gender	Source	PEN	FOX	KAN	TOB	GEN	ERY	CLI	TET	CIP	FUS	RFA
SSA1	9/2/18	Female	Nose Abscess	R	R	R	S	S	R	R	S	R	S	S
SSA4	1/4/18	-	Skin Carbuncle	R	S	S	S	S	S	S	S	S	S	S
SSA5	20/02/2018	Female	Pustule	R	R	R	S	S	S	S	R	S	R	S
SSA6	23/02/2018	Male	Bronchial Aspirate	R	S	S	S	S	S	S	S	S	S	S
SSA7	26/02/2018	Female	Wound	R	S	S	S	S	S	S	S	S	R	S
SSA8	30/03/2018	Female	Wound	R	R	S	S	S	R	R	R	S	R	S
SSA9	17/03/2018	Male	Sputum	R	S	S	S	S	S	S	S	S	S	S
SSA10	22/03/2018	Male	Wound	R	S	S	S	S	S	S	S	S	S	S
SSA11	3/4/18	Male	Wound	R	R	R	R	R	S	S	S	R	R	S
SSA12	14/04/2018	Male	Wound	R	S	S	S	S	S	S	S	S	R	S
SSA13	12/4/18	Male	Wound	R	R	R	R	R	S	S	S	S	R	S
SSA14	26/04/2018	Male	Urine	R	S	S	S	S	S	S	S	S	R	S
SSA15	23/04/2018	Male	Nose Abscess	R	R	S	S	S	S	S	S	S	S	S
SSA16	18/04/2018	Male	Blood	R	R	S	S	S	S	S	S	S	S	S
SSA17	28/04/2018	Female	Wound	R	R	S	S	S	S	S	S	I	S	S
SSA18	27-04-2018	Female	Wound	S	S	S	S	S	S	S	S	S	S	S
SSA19	29/05/2018	Male	Sputum	R	S	S	S	S	S	S	S	S	S	S
SSA20	24/05/2018	Female	Abscess	R	S	S	S	S	S	I	S	S	S	S
SSA21	18/05/2018	Female	Wound	S	S	S	S	S	S	S	S	S	R	S
SSA22	28/05/2018	Female	Sputum	S	S	S	S	S	S	S	S	R	S	S
SSA24	24/07/2018	Female	Wound	R	R	S	S	S	S	S	S	R	R	S
SSA25	19/07/2018	Female	Nose	S	S	S	S	S	S	S	S	S	S	S
SSA26	22/06/2018	Male	Wound	R	S	R	S	S	S	S	S	S	S	S
SSA27	16/07/2018	Female	Wound	R	R	R	R	R	S	S	R	S	R	S
SSA28	26/06/2018	Female	Wound	S	S	S	S	S	S	S	S	S	S	S
SSA29	13/07/2018	Female	Wound	R	S	S	S	S	S	S	S	S	S	S
SSA30	9/7/18	Female	Wound	R	S	S	S	S	S	S	S	S	S	S
SSA31	1/4/18	Female	Nose	R	R	S	S	S	S	S	S	S	R	S
SSA32	14/08/2018	Female	Nose	R	S	S	S	S	S	S	S	S	R	S
SSA33	16/08/2018	Male	Drainage Tip	R	R	R	R	R	R	R	R	R	R	R
SSA34	20/08/2018	Male	Urine	R	R	S	S	S	S	S	S	S	S	S

Penicillins
Cephalosporins
Aminoglycosides
Macrolides+Lincosamides
Tetracyclines
Fluoroquinolones
Fusidic acid
Rifampicin

R= Resistant, S= Sensitive, PEN= Penicillin, FOX= Cefoxitin, KAN= Kanamycin, TOB= Tobramycin, GEN= Gentamicin, ERY= Erythromycin, CLI= Clindamycin, TET= Tetracycline, CIP= Ciprofloxacin, FUS= Fusidic acid, RFA= Rifampicin.

3.2 Resistance genes

The antibiotic susceptibility testing results were further confirmed *in silico* using CARD and ResFinder v1.2. We were able to detect 15 different genes that confer resistance to the different tested antimicrobial agents including penicllin,

aminoglycosides, macrolides, tetracyclines, fusidic acid as well as Fosfomycin (**Table 7**).

The gene conferring resistance to penicillin, *blaZ,* was the most common being detected in twenty-four of the isolates (77.41%). Out of the 31 isolates, fourteen (45.16%) carried the *mecA* gene encoding for methicillin resistance. Among the seven (22.58%) isolates that carried one or more genes conferring resistance to aminoglycosides, three were positive for *aad(6)-Ia*, five for *aph(3')-IIIa* and four isolates had the *aac(6')-le-aph(2'')-la*. Additionally, we looked for the genes mediating resistance to macrolides, and detected *erm*(C) in two isolates (6.45%) and *erm*(A) in one (3.22%). Two different genes mediating resistance to trimethoprim were also detected. Three isolates carried the *dfrG* gene (9.67%), while only one isolate (3.22%) had the *dfrC* gene. Three different genes coding for tetracycline resistance (*tet45, tetK* and *tetM*) were detected in four of the isolates (12.9%), with *tetK* being the most common (2/31; 6.45%).

In addition, mutations in the quinolone resistance determining region (QRDR), *gyrA* and *parC,* were detected in seven (22.58%) of the isolates. The two most common mutations were Ser84Phe and Ser80Phe found in *gyrA* and *parC*, respectively. Seven distinct mutations were also found in *murA* and *glpT* genes mediating Fosfomycin resistance in seven (22.58%) of the isolates. The most common mutation found in *murA* led to an amino acid substitution at position 100 (A100V) in the target gene, whereas the most commonly detected mutation in *glpT* led to an amino acid substitution at position 291 (E291D). Mutations in *fusA* (L461K and V90I) were also detected conferring resistance to fusidic acid in three (9.67%) of the isolates, and mutations in *rpoB* (H481N, A473T and A477T) were also detected conferring resistance to rifampicin in one isolate (SSA33) (3.22%) (Table 8).

Screening for plasmids content was through using Plasmid Finder (Carattoli et al. 2014). Six out of the 31 sequenced genomes (19.35%) did not carry any plasmids. There were nine detected *rep* types within the isolates, including: *rep5*, *rep7, rep10, rep16*, *rep20, rep21, rep22, rep24* and *repUS5*, with *repUS5* found only in SSA20. *rep17* was the most commonly found *rep* type in the isolates carrying plasmids (52%; 13/25), followed by *rep16* and *rep5* which always co-existed (48%; 12/25) (Table 7).

Table 7: Types and distribution of resistance determinants and plasmid typing results. in silico analysis was done using CARD and ResFinder v1.2, while plasmid rep typing was done in silico using PlasmidFinder 1.3.

Isolate	Beta-lactam		Fosfomycin	Trimethoprim		Aminoglycoside			Macrolide				Tetracycline			Replicon Type
	mecA	*blaZ*	*FosB*	*dfrG*	*dfrC*	*aad(6)-Ia*	*APH(3')-IIIa*	*AAC(6')-Ie-APH(2'')-Ia*	*ermC*	*ermA*	*fusB*	*fusC*	*tet(K)*	*tet(M)*	*tet(45)*	
SSA1	■	■				■	■		■							*rep10, rep7, rep20*
SSA4		■	■	■												*rep16, rep5*
SSA5	■	■				■	■				■		■			*rep7, rep20*
SSA6		■														*rep16, rep5, rep7*
SSA7		■										■				*rep16, rep5, rep7*
SSA8	■	■							■		■		■			*rep10, rep7, rep20*
SSA9		■	■													*rep16, rep5*
SSA10		■		■												*rep16, rep5*
SSA11	■	■						■								*rep7, rep21*
SSA12		■	■								■					none
SSA13	■	■	■					■				■				none
SSA14	■	■	■									■				*rep7*
SSA15	■															none
SSA16	■															none
SSA17	■	■														*rep16, rep5*
SSA18																*rep7*
SSA19		■	■													*rep16, rep5*
SSA20		■	■	■												*repUS5*
SSA21												■				*rep7*
SSA22																*rep16, rep5*
SSA24	■	■														none
SSA25																*rep20, rep5*
SSA26		■	■				■									*rep16, rep5*
SSA27	■	■					■	■				■			■	*rep16, rep22, rep5, rep7*
SSA28																*rep7*
SSA29		■														*rep16, rep5, rep19*
SSA30		■														*rep20, rep24, rep7*
SSA31	■	■										■				*rep20, rep5*
SSA32		■										■				*rep16, rep5, rep7*
SSA33	■	■	■			■	■	■		■				■		*rep20, rep21*
SSA34	■	■			■											none

Table 8: Mutations that confer resistance to fluoroquinolones found in the quinolone resistance determining regions (QRDR) of *gyrA* and *parC*, fusidic acid (*fusA*), Fosfomycin (*glpT* and *murA*) and rifampicin (*rpoB*).

	Synonymous substitution					
Strain	**Quinolone resistance determining regions (QRDR)**		**Fusidic acid**	**Fosfomycin**		**Rifampicin**
	GyrA	**ParC**	**FusA**	**GlpT**	**murA**	**rpoB**
SSA1	S84L	S80F	-	-	-	-
SSA9	-	-	-	A100V, V213I	D278E, E291D	-
SSA10	-	-	-	A100V	E291D, T396N	-
SSA11	E88K	S80F	L461K	-	-	-
SSA12	-	-	-	L27K, A100V	E291D, T396N	-
SSA13	-	-	-	L27K, A100V	E291D, T396N	-
SSA17	-	S80F	-	-	-	-
SSA19	-	-	-	A100V, V213I	D278E, E291D	-
SSA20	-	-	-	L27K, A100V	E291D, T396N	-
SSA22	S84L	S80F	-	-	-	-
SSA24	S84L	S80F	V90I	-	-	-
SSA26	-	S80F	-	-	-	-
SSA33	S84L	s80F	L461K	-	G257D	H481N, A473T, A477T

3.3 MLST, *spa* and SCC*mec* Typing

3.3.1 MLST and *spa* Typing

MLST and *spa* typing were performed for sequence based molecular typing of the isolates. An *in silico* MLST analysis was performed using seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi and yqiL*). A total of 15 different STs including a novel ST was detected. ST80 was the most common type (22.58%; 7/31), followed by ST6 (12.9%; 4/31), ST1 (12.9%; 4/31), ST121 (9.67%; 3/31), ST22 (9.67%; 3/31), and a singleton (3.22%; 1/31) were detected (**Table 9**). SSA19 had a novel allelic profile which was 2-2-2-349-6-3-2, and was a single locus variant of ST30.

In total, 19 different *spa* types were detected among the 31 isolates. *spa* t044 was the most common *spa* type detected in 22.58% (7/31) of the isolates, followed by t127 (12.9%; 4/31), t304 (9.67%; 3/31), t314 (6.45%;2/31) while the remaining were either detected once each or was untypeable (SSA25). Combining the MLST and *spa* typing results revealed that the common types were ST80-t044 (19.35%; 6/31), ST1-t127 (12.9%; 4/31), ST6-t304 (9.67%; 3/31) and ST121-t314 (6.45%; 2/31), with the ST1 being linked to *spa* t127 (100%; 4/4), ST6 to *spa* t304 (75%; 3/4) and ST80 to *spa* t044 (85.71%; 6/7).

Table 9: Overview of *S. aureus* ST and *spa* types.

Isolate	Loci alleles							ST	*spa*
	arcC	*aroE*	*glpF*	*gmk*	*pta*	*tpi*	*yqiL*		
SSA1	1	3	1	14	11	51	10	80	t044
SSA4	13	13	1	1	12	11	13	15	t360
SSA5	1	3	1	14	11	51	10	80	t044
SSA6	12	4	1	4	12	1	3	6	t701
SSA7	1	1	1	1	1	1	1	1	t127
SSA8	1	3	1	14	11	51	10	80	t044
SSA9	2	2	2	2	6	3	2	30	t012
SSA10	46	75	49	44	13	68	60	152	t355
SSA11	1	3	1	14	11	51	10	80	t044
SSA12	6	5	6	2	7	14	5	121	t2155
SSA13	6	5	6	2	7	14	5	121	t314
SSA14	1	4	1	8	4	4	3	72	t3468
SSA15	12	4	1	4	12	1	3	6	t304
SSA16	12	4	1	4	12	1	3	6	t304
SSA17	12	4	1	4	12	1	3	6	t304
SSA18	1	3	1	14	11	51	10	80	t044
SSA19	2	2	2	349	6	3	2	Novel	t018
SSA20	6	5	6	2	7	14	5	121	t314
SSA21	1	1	1	1	1	1	1	1	t127
SSA22	22	1	14	220	12	4	31	2884	t2393
SSA24	7	6	1	5	8	8	6	22	t651
SSA25	7	6	1	5	8	8	6	22	Untypable
SSA26	13	13	1	1	12	1	13	199	t279
SSA27	1	1	1	1	1	1	1	1	t127
SSA28	1	3	1	14	11	51	10	80	t044
SSA29	3	1	1	8	1	1	1	188	t189
SSA30	1	3	1	14	11	51	10	80	t10733
SSA31	1	4	1	4	12	41	10	149	t002
SSA32	1	1	1	1	1	1	1	1	t127
SSA33	2	3	1	1	4	4	3	239	t037
SSA34	7	6	1	5	8	8	6	22	t1328

3.3.2 SCC*mec* Typing

Typing of the *mec* cassette revealed that 57.14% of the MRSA isolates (8/14) carried SCC*mec* type IV(2B), and only one isolate (SSA33) had the SCC*mec* type III. We could not identify the *mec* cassette in five of the isolates. We used an *in silico* approach through the SCCmecFinder and determined the type of the cassette in three out of the five untypeable cassettes (SSA13, SSA14 and SSA27), and which were of type V(5C2) but with less than 40% sequence coverage. When compared to the SCC*mec* type V(5C2) elements of a reference MRSA JCSC3624 (Genbank: AB121219.1), all three isolates (SSA13, SSA14 and SSA27) carried *ccrC1*, *mecA* genes and the *IS431* sequence that are linked to SCC*mec* typeV (5C2), while lacking the conserved *hsdR, hsdS* and *hsdM* genes of the cassette. SSA27 on the other hand, also carried another *ccr* gene complex: class 5 (*ccrA1* and *ccrB1)* (**Figure 1**).

SCC*mec* type IV(2B) cassette distinguished by having *mecR1, mecA, IS1272, ccrA* and *ccrB*, was detected in SSA11 and SSA34 showing complete sequence similarity to *mecA* and *mecR1*. However, we could not detect the *ccr* gene complex (*ccrA* and *ccrB*) and *IS1272* in SSA11 and SSA34, respectively (**Figure 1**).

Combining the MLST and the *mec typing* results revealed that the common types were ST80-t044 (19.35%; 6/31) with 50% being ST80-t044-IV, ST1-t127 (12.9%; 4/31) with one being ST1-t127-V-like, ST6-t304-IV (9.67%; 3/31) and ST121-t314 (6.45%; 2/31) with one being ST121-t314-V-like.

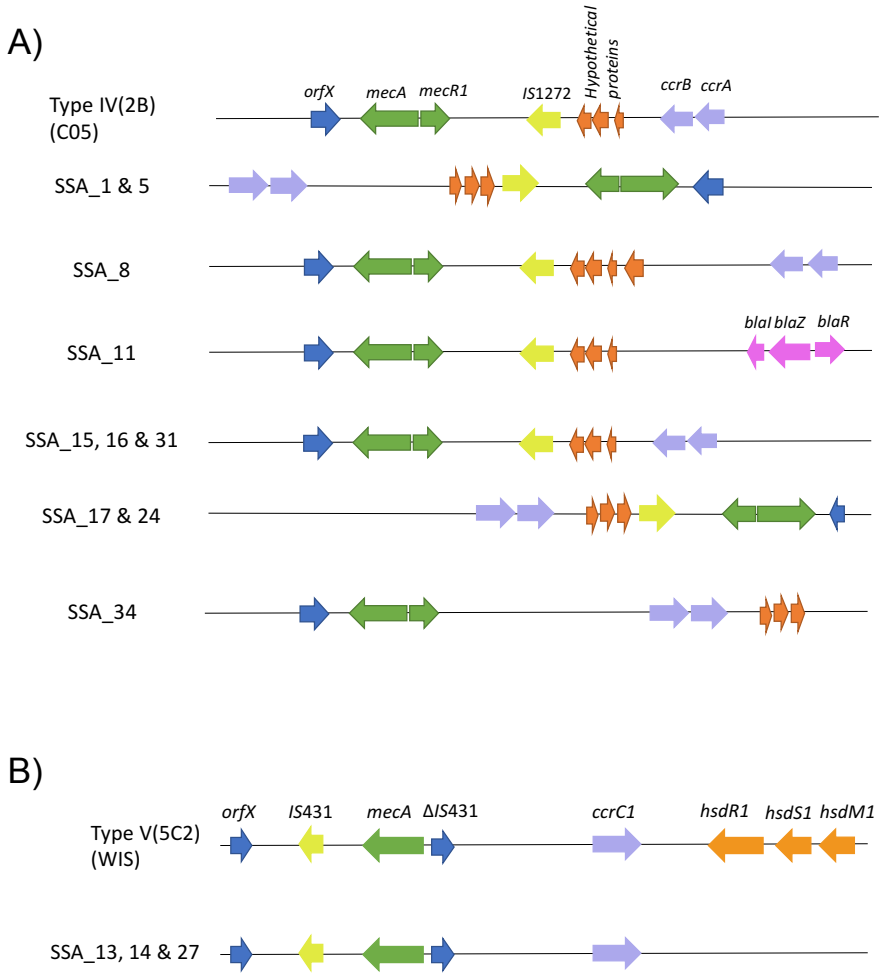


Figure 1: A) Comparison of the SCC*mec* elements of the MRSA isolates in this study carrying SCC*mec* type IV(2B) with that of a reference MRSA isolate C05 (GenBank: AB425824). B) Comparison of the SCC*mec* elements of MRSA isolates that carried SCC*mec* type V(5C2) with that of a reference MRSA isolates WIS (GenBank: AB121219). The colors and arrows indicate the gene and transcription direction.

3.4 PFGE

Pulse-field Gel electrophoresis was performed to determine the genetic relatedness between the different isolates (**Figure 2**). A total of 20 distinct pulsotypes (PTs) were identified. Isolates that had a three or more bands difference were considered as having different PFGE pattern, according to Goering and Winters (1992). Most isolates having the same ST, or single, or double locus variants (SLVs or DLVS, respectively) were clustered together. Nevertheless, some isolates, such as SSA1 (ST-80), did not cluster with others having the same ST or *spa* type.

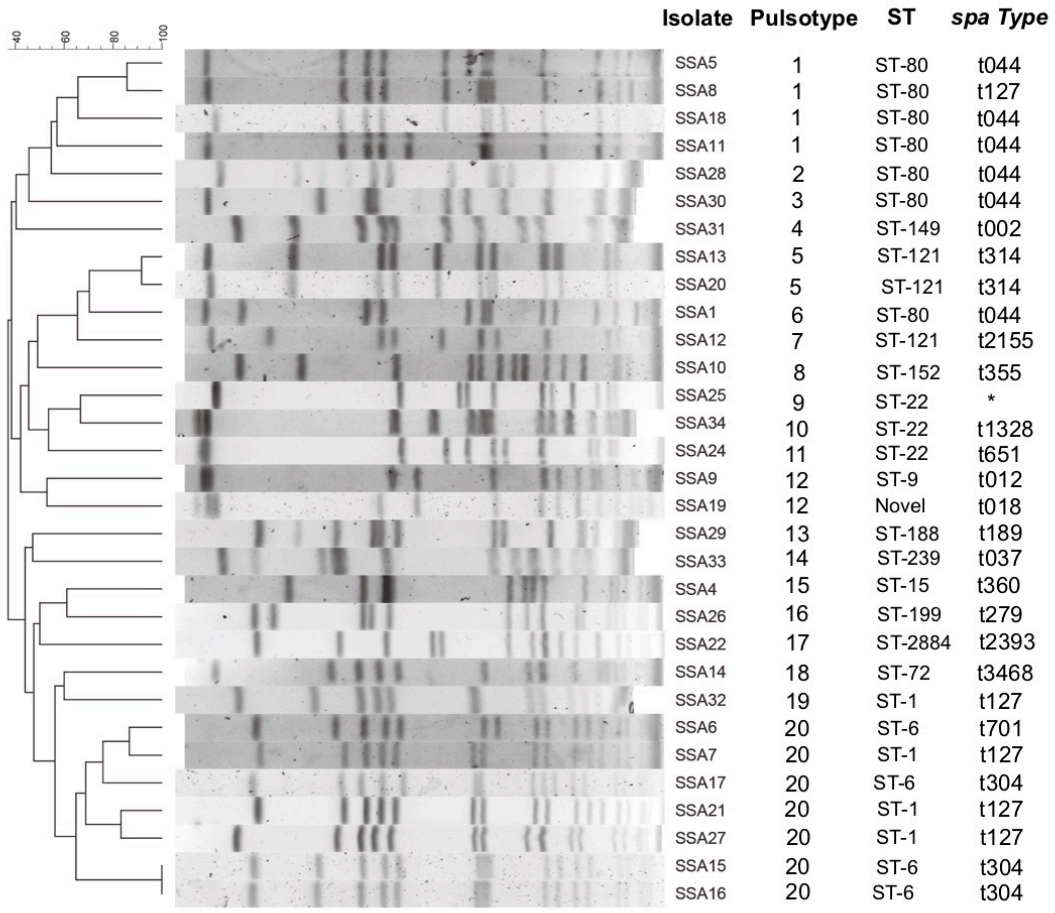


Figure 2: PFGE profiles, Pulsotypes, ST and *spa* type of S. aureus isolates. Dendogram generated by BioNumerics software version 7.6.1 showing the relationship of the isolates based on their banding patterns generated by *SmaI* restriction digestion.

3.5 *agr* Typing and Biofilm Production

Using Blast we were able to determine the *agr* types. *agr* type III was the most common being detected in 45.16% (14/31) of the isolates, followed by *agr* type I in 35.48% (11/31) of the isolates. The remaining isolates had *agr* type II (9.67%; n= 3/31) and *agr* type IV (9.67%; n= 3/31) (**Table 10**). Biofilm formation was determined using a qualitative assay. Almost half of the isolates (45.16%; n= 14/31) showed a good potential to be engaged in biofilm formation.

Table 10: Biofilm production and *agr* type of S. aureus isolates. A scale of 1-3 was set for the isolates biofilm production ability, with 1 for weak biofilm production, 2 for moderate biofilm production and 3 for strong biofilm production.

Samples	Biofilm production	agr type
SSA1	1	III
SSA4	1	II
SSA5	1	III
SSA6	1	I
SSA7	2	III
SSA8	1	III
SSA9	1	III
SSA10	1	I
SSA11	2	III
SSA12	1	IV
SSA13	1	IV
SSA14	1	I
SSA15	1	I
SSA16	1	I
SSA17	1	I
SSA18	1	III
SSA19	3	III
SSA20	2	IV
SSA21	3	III
SSA22	3	III
SSA24	3	I
SSA25	3	I
SSA26	3	II
SSA27	3	III
SSA28	3	III
SSA29	3	I
SSA30	3	III
SSA31	3	II
SSA32	3	III
SSA33	3	I
SSA34	3	I

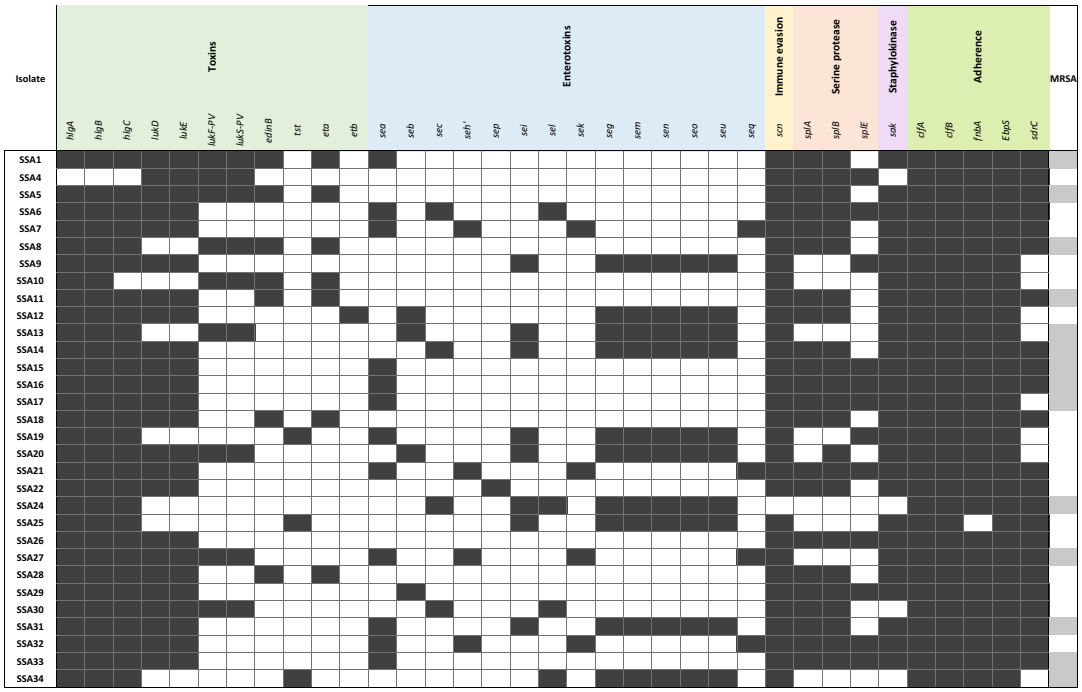
3.6 Virulence genes

We screened for the presence/absence of 26 virulence factors (VFs), including: Panton-Valentine leukocidin (*pvl)*, staphylococcal enterotoxin genes (*entA, entB, entC, entH, entG* and *entD*), the exfoliative toxin genes (*eta*, *etb*), hemolysin genes (*hla*, *hlb*), adhesion factor genes (*fnbA*, *clfA* and *clfB*) as well as proteases and the immune evasion gene *scn.* Screening was done *in silico* using the VirulenceFinder 1.2 tool available on CGE and RAST (Joensen et al. 2014).

clfA, clfB and *fnbA* were present in all the isolates except for SSA25, which was negative for *fnbA*. Among the 31 isolates analyzed, 74.19% (23/31) isolates harboured at least one of the fourteen staphylococcal enterotoxin (SE) genes. The gene *sea* was the most prevalent SE gene (38.7%; 12/31) among the isolates, followed by the *egc* cluster genes (*seg, sem, sen, seo* and *seu)* (32.25%; 10/31) and *sei* (25.8%, 8/31). A common enterotoxin gene profile, namely *sea-seh-sek-seq*, was observed among the ST121 isolates.

The genes *eta* and *etb* were detected in 25.8% (7/31) of the isolates. Three isolates (9.67%) (SSA19, SSA25 and SSA34) carried *tst-1*, the gene coding for the toxic shock syndrome toxin (TSST-1*)*. Nine (29%) *lukS/F*-PV-positive *S. aureus* isolates were identified with more than half being MRSA.

Table 11: Virulence factors detected *in silico* using CGE and RAST.



3.7 Phages

Using PHASTER annotation tool, differences in the phage content between the isolates were determined. In total, 16 different intact phages were identified. *Staphylococcus* phage JS01 (accession # NC_021773) was the most common, being identified in 48.38% (15/31) of the isolates. Phage JS01 was found in all ST121 and ST6 isolates and in 85.71% (6/7) of the ST80 *S. aureus*. It mainly carried genes coding for phage proteins, hypothetical proteins, DNA replication and repair proteins and it also coded for the staphylokinase virulence factor. *Staphylococcus* Phi7401PVL (accession # NC_020199) was present in 12.9% (4/31) of the isolates, all of which were PVL-positive ST80 *S. aureus* (n= 4/31). Phage PT1028 was also detected and three of the isolates were typed as ST121. (**Table 12**).

Table 12: Phages content of the 31 sequenced genomes as inferred by the phage search tool PHASTER (Arndt et al. 2016).

Strain	Size	Completdness	# total protein	Phage name	GC	ST- type	PVL (+) OR (-)
SSA1	33Kb	intact	36	phi7401PVL	33.32%	ST80	+
	33.8Kb	intact	40	phiMR11	35.22%		
	39.5Kb	intact	60	JS01	33.75%		
SSA4	59.4Kb	intact	74	phiSa119	33.04%	ST795	+
SSA5	39.5Kb	intact	60	JS01	33.75%	ST80	+
	52.1Kb	intact	57	phi7401PVL	33.16%		
SSA6	51.5Kb	intact	68	JS01	32.65%	ST6	-
SSA7	59.3Kb	intact	70	tp310_1	32.24%	ST1	-
SSA8	51.1Kb	intact	57	phi7401PVL	33.19%	ST80	+
	39.5Kb	intact	60	JS01	33.76%		
SSA9	57.6Kb	intact	73	phi2958PVL	33.25%	30	-
SSA10	74.5Kb	intact	75	phi2958PVL	32.86%	ST152	+
	46.3Kb	intact	68	phiNM3	33.05%		
SSA11	30.8Kb	intact	47	JS01	34.22%	ST80	-
	39.7Kb	intact	56	YMC/09/04/R1988	33.82%		
	30.9Kb	intact	43	53	34.61%		
SSA12	50.7Kb	intact	65	B236	34.69%	ST121	-
	41.5Kb	intact	38	PT1028	32.33%		
	56.2Kb	intact	71	JS01	32.76%		
SSA13	54.4Kb	intact	68	JS01	32.70%	ST121	+
	47.6Kb	intact	60	11	34.71%		
	19.2Kb	intact	26	PT1028	31.35%		
SSA14	-	-	-	-	-	ST72	-
SSA15	28.8Kb	intact	38	JS01	33.71%	ST6	-
	52.8Kb	intact	61	YMC/09/04/R1988	33.46%		
	20.8Kb	intact	35	96	35.87%		
SSA16	23.5Kb	intact	38	96	35.58%	ST6	-
	28.8Kb	intact	38	JS01	33.71%		
	44.4Kb	intact	62	YMC/09/04/R1988	33.57%		
SSA17	51.5Kb	intact	67	JS01	32.65%	ST6	-
	48.7Kb	intact	73	B236	34.22%		
SSA18	55.7Kb	intact	65	JS01	32.52%	ST80	-
SSA19	33.8Kb	intact	34	PT1028	32.57%	Novel	-
	53.2Kb	intact	65	phiNM3	32.56%		
SSA20	54.4Kb	intact	66	JS01	32.70%	ST121	+
	41.5Kb	intact	37	PT1028	32.35%		
SSA21	59.3Kb	intact	70	tp310_1	32.24%	ST1	-
SSA22	-	-	-	-	-	ST2884	-
SSA24	48.6Kb	intact	64	phi2958PVL	33.10%	ST22	-
SSA25	-	-	-	-	-	ST22	-
SSA26	53.2Kb	intact	67	11	34.19%	ST199	-
SSA27	60.9Kb	intact	77	phi2958PVL	32.73%	ST1	+
	55.8Kb	intact	61	tp310_1	32.10%		
SSA28	55.7Kb	intact	66	JS01	32.52%	ST80	-
SSA29	45.8Kb	intact	66	phiNM3	33.00%	ST188	-
SSA30	56.5Kb	intact	64	phiN315	32.14%	ST80	+
	59.6Kb	intact	69	phi7401PVL	32.80%		
	50.9Kb	intact	66	88	34.82%		
SSA31	57.5Kb	intact	73	JS01	32.26%	ST149	-
	61.8Kb	intact	77	phi2958PVL	32.81%		
SSA32	28.6Kb	intact	37	JS01	33.61%	ST1	-
	31Kb	intact	41	53	34.72%		
	61.6Kb	intact	69	phi2958PVL	32.92%		
SSA33	-	-	-	-	-	ST239	-
SSA34	25.2Kb	intact	32	B166	35.75%	ST22	-

3.8 Pan-genome Analysis

Pan-genome analysis of the isolates revealed the presence of a total of 4679 unique protein coding sequences. Genes identified as being part of the core genome constituted 1982 coding sequences. Moreover, genes present in 15-95% of the isolates were designated as being shell and constituted 895 protein coding sequences, while those present in <15% constituted 1712 protein coding sequences.

Maximum likelihood tree based on the pan-genome analysis of the coding regions in *S. aureus* showed the clustering of the isolates having the same MLST type (ST-80, ST-1, ST-6, ST-121 and ST-22).

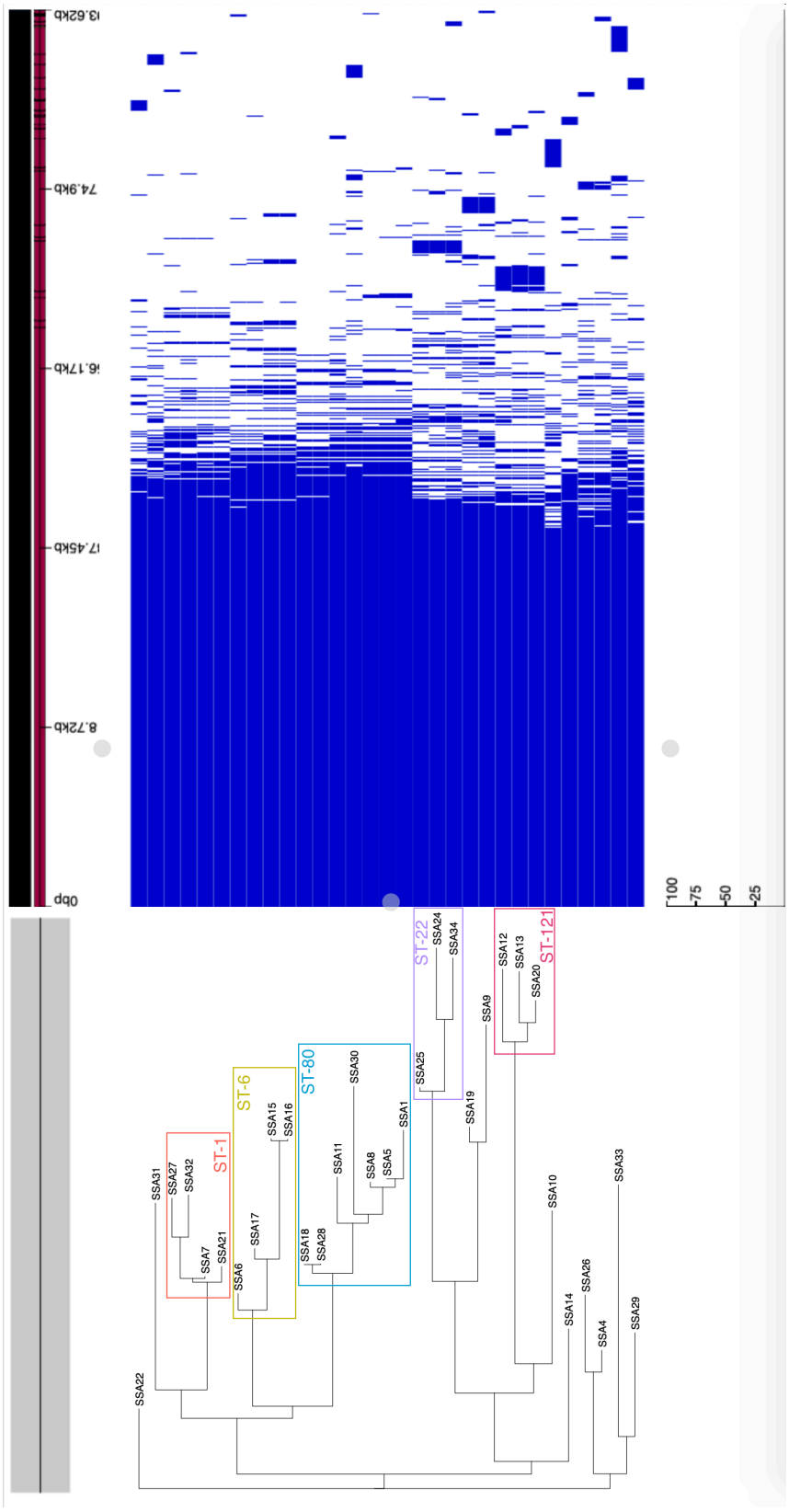


Figure 3: Presence/Absence matrix with its associated maximum likelihood phylogenetic tree based on pan genome analysis of coding regions in *S. aureus* isolates.

Chapter Four

Discussion

S. aureus is a common human pathogen that can cause a plethora of infections. It was associated with a high-rate of both hospital and community acquired infections (Lakhundi and Zhang, 2018). The ability of *S. aureus* to evade the immune system and cause damage to host cells was attributed to its diverse genetic composition and its ability to acquire virulence determinants (Turner, N.A., 2019). Due to the difficulty in treating multi-drug resistant *S. aureus,* prevalence of MRSA in Lebanon (30% to 72%) has brought about many concerns (Harastani et al., 2014; Tokajian et al., 2010). The aim of this study was to conduct a detailed whole-genome based molecular characterization of 31 representative clinical *S. aureus* isolates recovered in 2018 from Lebanon.

In the current study, methicillin resistance was observed in 45.16% (14/31) of the *S. aureus* isolates. The frequency of MRSA isolates in this study is higher than that previously reported in Lebanon by Harastani et al. (2014) but lower than the percentage stated in previous reports by Tokajian et al. 2010. This difference in MRSA rates in clinical isolates from Lebanon in the different studies could be due to the difference in regions, time period, and the different infection and prevention programs implemented by hospitals. According to the antibiotic susceptibility test results, 32.25% of the isolates were MDR, all of which were MRSA strains, and which was in accordance with previous data from Lebanon (Harastani et al., 2014). The MRSA isolates were characterized with a higher resistance rate than MSSA isolates for aminoglycosides, macrolides, tetracyclines, ciprofloxacin and fusidic

acid. These results are in accordance with previous studies that reported on MRSA isolates being in general more resistant to different antimicrobial agents than MSSA (Thompson and Brown, 2014; Chen et al., 2014).

Among the MRSA isolates that carried the *mecA* gene, 50% belonged to ST80 and ST6 (28.57% and 21.45%, respectively), followed by ST22 (14.28%) with the remaining 35.67% consisting of one isolate from each of the following MLST types; ST1, ST121, ST72, ST239 and ST149. The predominance of ST80-MRSA in Lebanon and in the Middle East was also previously reported (Harastani et al., 2014; Khalil, Hashwa, Shihabi and Tokajian, 2012). The worldwide dissemination of the MRSA-ST80 clone however, decreased over the past few years (Mairi, Touati and Lavigne, 2020). Nevertheless, we reported the dominance of MRSA-ST80 strains in Lebanon at a level similar to the previous report from Lebanon (25.6%) (Tokajian et al., 2011). The percentage of MRSA-ST80 isolates detected in Lebanon was higher than that reported in Oman (4%), Turkey (4%) and in United Arab Emirates (19%) but was similar to that seen in Palestine (31%) (Oksuz et al., 2013; Udo, Al-Lawati, Al-Muharmi and Thukral, 2014; Sonnevend et al., 2011; Laham et al., 2015).

Aminoglycosides are broad-spectrum antibiotics that are used for the treatment of staphylococcal infections. Aminoglycosides are prescribed in combination with either ß-lactams or glycopeptides for the treatment of complicated *S. aureus* infections (Seyedi-Marghaki et al., 2019). Aminoglycoside-modifying enzymes (AME) are the main mechanism of resistance against aminoglycosides. The most important of these enzymes are aminoglycoside-6'-*N*-acetyltransferase/2"-*O*-phosphoryltransferase ([AAC (6')-APH(2")]; aminoglycoside-3′-*O*-phosphoryltransferase III [APH(3′)-III] and aminoglycoside-4′-*O*-phosphoryltransferase I [ANT (4′)-I], modifying different aminoglycosides including: gentamicin, kanamycin and tobramycin, respectively (Ida

et al., 2001). Several studies in the Middle East reported the high incidence of aminoglycoside resistance in *S. aureus* isolates, especially in MRSA (Yadegar, Sattari, Mozafari and Goudarzi, 2009; Emaneini et al., 2013). In line with that, our results showed a slight increase in gentamicin resistance in *S. aureus* isolates (12.9%) compared to previous reports from Lebanon (9%) (Tokajian et al., 2011). Out of the seven *S. aureus* isolates that carried genes conferring resistance to aminoglycosides, 86% (6/7) were MRSA isolates, with the resistance being linked to three genes *aph(3')-IIIa, aac(6')-Ie-aph(2")* and *aad(6)-Ia* and mainly seen in the ST80-t044 MRSA isolates. Goudarzi et al. (2020) showed similar findings in ST80-MRSA isolates in Iran. Given the alarming rate of resistance to aminoglycoside among MRSA, the monitoring of aminoglycoside resistance and AME genes should be performed to reduce treatment failure in patients with staphylococcal infections.

The genetic basis of fusidic acid resistance in *S. aureus* is the outcome of acquiring the *fusB* or *fusC* genes or from mutations in the *fusA* gene. One of the most prevalent mutations conferring resistance to fusidic acid in clinical isolates, L461K in *fusA* (Castanheira et al., 2010), was detected in SSA11 and SSA33. It is noteworthy that all the ST1 isolates (n= 4/31) in this study were resistant to fusidic acid and was attributed to the *fusC* gene. In Denmark and in New Zealand, ST1 *S. aureus* isolates were commonly detected and were also positive for the *fusC* gene (Edslev et al., 2017; Baines et al., 2016). It is worth noting that 92% (12/13) of the isolates that showed fusidic acid resistance were related to skin and soft tissue infections. These findings could indicate possible clonal selection for *fusC*-positive ST1 *S. aureus* in Lebanon knowing that fusidic acid is commonly used to treat different skin infections (https://www.moph.gov.lb).

On the other hand, we detected six distinct mutations in *glpT* and *murA* and that conferred resistance to Fosfomycin. Our results showed that the ST121 isolates had common *glpT* and *murA* mutations (*glpT*: L27K, A100V; *murA*: E291D, T396N), with all additionally having the *fosB* gene. *glpT* and/or *uhpT* mutants previously detected in China were common in isolates belonging to ST5, which was the predominant sequence type among the Fosfomycin-resistant MRSA isolates (Wu et al., 2018; Fu et al., 2016).

We further analyzed the SCC*mec* cassette in all the MRSA isolates in this study. The common type was SCC*mec* type IV(2B) (n = 8), followed by SCC*mec* type III (n = 1). Tokajian et al. (2010) reported that SCC*mec* type IV was the major SCC*mec* carried by MRSA isolates (88%) in Lebanon, which was consistent with our results and indicated the epidemicity of those isolates. However, we also detected SCC*mec* type III in one isolate (SSA33). SCC*mec* type III is commonly found in *S. aureus* isolate linked to hospital acquired infections (Asghar, 2014). SSA33 was a MRSA isolate and resistant to all other tested antibiotics and was typed as ST239-t037. Harastani et al. (2014) also detected only one ST239-t037 isolate that carried SCC*mec* type III cassette and was also resistant to all tested antimicrobial agents.

On the other hand, we had untypeable SCC*mec* cassettes harbouring multiple *ccr* complexes or missing some of the conserved genes. Using the SCCmecFinder, we were able to predict the *mec* cassettes of SSA11 and SSA34 to be of type IV(2B). Although SSA11 (ST80) carried *IS1272,* it lacked the recombinase coding genes (*ccrA* and *ccrB)*. It harboured a pseudo-SCC*mec* element resembling that usually detected in SCC*mec* type IV(2B). A recent study also showed that a MRSA ST72 isolate recovered from South Korea harboured a similar pseudo-SCC*mec* element. Yoon et al. (2019) consequently suggested that the loss of the *crr* complex could be a mechanism

used by MRSA isolates to adapt in an antimicrobial-abundant habitat by having an immobile *mecA* gene. Moreover, SSA34 (ST22) was predicted to carry the type IV(2B) SCC*mec*, however SSA34 lacked the *IS*1272 type IV associated sequence. In addition, it harboured the transposon *Tn552,* which carried the *bla* operon, downstream of the predicted type IV(2B)-like SCC*mec* element. To our knowledge, this is the first report of such SCC*mec* element. Further investigations are needed to further characterize it.

SSA13, SSA14, and SSA27 had the type V SCC*mec* element, which was previously detected in *S. aureus* isolates recovered from Lebanon (Harastani et al., 2014). However, the studied cassettes showed less than 40% sequence similarity to the reference type V element; *hsdR, hsdM* and *hsdS* genes could not be detected in all the three, while SSA27 had an extra *crr* complex.

Based on MLST typing results, we had 15 distinct sequence types, with one being a novel type. ST80 was the most common sequence type identified, again being consistent with previous results from Lebanon (Harastani et al., 2014). The other common STs were ST6 and ST1. The investigated MRSA and MSSA isolates in this study were assigned to 12 different *spa* types, which showed a diverse genetic profile. The most common *spa* type among MRSA isolates was t044 (28.57%) that mostly belonged to MRSA-ST80-IV (75%), followed by t304 (9.67%) in isolates that belonged to ST6-t304-IV. In accordance with what was previously reported in the region, MRSA ST80-t044-IV was the predominate MRSA clone among the isolates and it was mostly associated with skin infections (Tokajian et al., 2010). Consistent with what was reported in Oman and UAE, all three of the MRSA ST6-t304-IV isolates were PVL negative, indicating a possible expansion of the MRSA ST6-t304-IV clone in the Middle East (Udo et al. 2014; Sonnevend et al. 2012). Greater diversity

in *spa* types was observed in MSSA than MRSA isolate. MSSA isolates were associated with thirteen of the nineteen *spa* types detected, including t2155 which is reported for the first time in Lebanon. The t2155 *spa* type was associated with a ST121 strain. MSSA ST121-t2155 was reported by Gómez-Sanz et al. (2013) in Spain, this suggested expansion of the ST121 clone in Lebanon, as it was previously only linked to t314 and t159 in Lebanon and in the region (Tokajian et al. 2010; Boswihi, Udo and Al-Sweih, 2016).

We screened for the common virulence determinants in *S. aureus* using VirulenceFinder and BLAST. We looked for different toxins/enterotoxins, and the *eta* gene was the most common (38.7%; 12/31) and which was consistent with results obtained from isolates recovered in Jordan (Naffa et al., 2006). Ten (32.2%) of the isolates carried enterotoxin genes belonging to the enterotoxin gene cluster (*egc*), with most having the full cluster of genes (*seg, sei, sem, sen, seo* and *seu*). The ST1 isolates, where positive for *sea-seh-sek-seq*. This combination was also previously reported in *S. aureus* recovered from Japan and typed as ST81. ST81 is a single-locus variant of ST1 (Sato'o et al., 2014). Having *seh* only in ST1 isolates was in accordance with previous findings (Diep et al., 2006). However, it is noteworthy that the ST80, and similar to results from Tunis (Ben Nejma, Mastouri, Bel Hadj Jrad and Nour, 2013), had the least number of enterotoxins. None of the isolates had the *see* gene, which was also in accordance with previous reports from Lebanon and Jordan (Tokajian et al., 2011; Naffa et al., 2006). Additionally, only three isolates were *tst*-positive, which was also consistent with previous reports from Lebanon (Tokajian et al., 2011). Interestingly, SSA33 was *tst*-positive but yet MRSA (ST22-t1328/IV). *tst*-positive ST22-t1328/IV could be a variant of *tst*-positive ST22-IVa-t223 strain which was

previously detected in Kuwait, Jordan, Saudi Arabia and Egypt (Udo, Boswihi and Al-Sweih, 2016).

All four *agr* types were detected in this study. The most prevalent *agr* group was *agr* type III which was found in 45.16% (14/31) of the isolates, followed closely by *agr* type I 35.48% (11/31). This data is contrary to what was seen in Iran, where *agr* type I was the dominant (Javdan, Narimani, Shahini Shams Abadi and Gholipour, 2019). Isolates carrying *agr* type III or *agr* type IV were the ones having the highest number of toxin genes, each carrying on the average 9 to 13 genes, respectively. Previously, the distribution of toxin genes was found to be associated with the *agr* type of the strain (Jarraud et al., 2002). Jarraud et al. (2002) found that the *egc* cluster was relevant to *agr* type IV, while negatively correlating with *agr* type I. However, our results contrasted this finding as 4 of the 10 complete and incomplete *egc* clusters found in our isolates were linked to *agr* type I.

The current study provided a detailed whole-genome molecular characterization of thirty-one *S. aureus* isolates collected from Lebanon. Our findings identified high resistance profiles, revealed and confirmed the prevalence of MRSA in Lebanon. Further larger scale epidemiological studies are required along with additional infection prevention measures in hospital settings in order to follow and limit the spread of multi-drug resistant MRSA strains in Lebanon.

References

Al Laham, N., Mediavilla, J. R., Chen, L., Abdelateef, N., Elamreen, F. A., Ginocchio, C. C., Pierard, D., Becker, K., & Kreiswirth, B. N. (2015). MRSA clonal complex 22 strains harboring toxic shock syndrome toxin (TSST-1) are endemic in the primary hospital in Gaza, Palestine. *PloS one*, *10*(3), e0120008. https://doi.org/10.1371/journal.pone.0120008

Al-Mebairik, N., El-Kersh, T., Al-Sheikh, Y., & Marie, M. (2016). A review of virulence factors, pathogenesis, and antibiotic resistance in Staphylococcus aureus. *Reviews In Medical Microbiology*, *27*(2), 50-56. doi: 10.1097/mrm.0000000000000067

Andrews. (2010). Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data. Retrieved June 10, 2019, from https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., & Wishart, D. S. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research*, *44*(Web Server issue), W16–W21. https://doi.org/10.1093/nar/gkw387

Asghar A. H. (2014). Molecular characterization of methicillin-resistant Staphylococcus aureus isolated from tertiary care hospitals. *Pakistan journal of medical sciences*, *30*(4), 698–702. https://doi.org/10.12669/pjms.304.4946

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A. ... & Meyer, F. (2008). The RAST Server: rapid annotations using subsystems technology. BMC genomics, 9(1), 75.

Baines, S. L., Howden, B. P., Heffernan, H., Stinear, T. P., Carter, G. P., Seemann, T., Kwong, J. C., Ritchie, S. R., & Williamson, D. A. (2016). Rapid Emergence and Evolution of Staphylococcus aureus Clones Harboring fusC-Containing Staphylococcal Cassette Chromosome Elements. *Antimicrobial agents and chemotherapy*, *60*(4), 2359–2365. https://doi.org/10.1128/AAC.03020-15

Ben Nejma, M., Mastouri, M., Bel Hadj Jrad, B., & Nour, M. (2013). Characterization of ST80 Panton-Valentine leukocidin-positive community-acquired methicillin-resistant Staphylococcus aureus clone in Tunisia. *Diagnostic Microbiology And Infectious Disease*, *77*(1), 20-24. doi: 10.1016/j.diagmicrobio.2008.02.010

Bien, J., Sokolova, O., & Bozko, P. (2011). Characterization of Virulence Factors of Staphylococcus aureus: Novel Function of Known Virulence Factors That Are Implicated in Activation of Airway Epithelial Proinflammatory Response. *Journal of pathogens*, *2011*, 601905. https://doi.org/10.4061/2011/601905

Boakes, E., Kearns, A. M., Ganner, M., Perry, C., Hill, R. L., & Ellington, M. J. (2011). Distinct bacteriophages encoding Panton-Valentine leukocidin (PVL) among international methicillin-resistant Staphylococcus aureus clones harboring PVL.

Journal of clinical microbiology, *49*(2), 684–692. https://doi.org/10.1128/JCM.01917-10

Borg, M., de Kraker, M., Scicluna, E., van de Sande-Bruinsma, N., Tiemersma, E., Monen, J., & Grundmann, H. (2007). Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in invasive isolates from southern and eastern Mediterranean countries. *Journal Of Antimicrobial Chemotherapy*, *60*(6), 1310-1315. doi: 10.1093/jac/dkm365

Boswihi, S. S., Udo, E. E., & Al-Sweih, N. (2016). Shifts in the Clonal Distribution of Methicillin-Resistant Staphylococcus aureus in Kuwait Hospitals: 1992-2010. *PloS one*, *11*(9), e0162744. https://doi.org/10.1371/journal.pone.0162744

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., Olson, R., Overbeek, R., Parrello, B., Pusch, G. D., Shukla, M., Thomason, J. A., 3rd, Stevens, R., Vonstein, V., Wattam, A. R., & Xia, F. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific reports*, *5*, 8365. https://doi.org/10.1038/srep08365

Carattoli, A., Zankari, E., García-Fernández, A., Larsen, M. V., Lund, O., Villa, L., ... Hasman, H. (2014). In Silico Detection and Typing of Plasmids Using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrobial Agents and Chemotherapy*, *58*(7), 3895–3903. https://doi.org/10.1128/AAC.02412-14

Castanheira, M., Watters, A. A., Bell, J. M., Turnidge, J. D., & Jones, R. N. (2010). Fusidic acid resistance rates and prevalence of resistance mechanisms among Staphylococcus spp. isolated in North America and Australia, 2007-2008. *Antimicrobial agents and chemotherapy*, *54*(9), 3614–3617. https://doi.org/10.1128/AAC.01390-09

Chamoun, K., Farah, M., Araj, G., Daoud, Z., Moghnieh, R., Salameh, P., Saade, D., Mokhbat, J., Abboud, E., Hamze, M., Abboud, E., Jisr, T., Haddad, A., Feghali, R., Azar, N., El-Zaatari, M., Chedid, M., Haddad, C., Zouain Dib Nehme, M., Barakat, A., … Lebanese Society of Infectious Diseases Study Group (LSID study group) (2016). Surveillance of antimicrobial resistance in Lebanese hospitals: retrospective nationwide compiled data. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*, *46*, 64–70. https://doi.org/10.1016/j.ijid.2016.03.010

Chen, C., & Huang, Y. (2014). New epidemiology of Staphylococcus aureus infection in Asia. *Clinical Microbiology And Infection*, *20*(7), 605-623. doi: 10.1111/1469-0691.12705

Dukic, V. M., Lauderdale, D. S., Wilder, J., Daum, R. S., & David, M. Z. (2013). Epidemics of community-associated methicillin-resistant Staphylococcus aureus in the United States: a meta-analysis. *PloS one*, *8*(1), e52722. https://doi.org/10.1371/journal.pone.0052722

Edslev, S. M., Clausen, M. L., Agner, T., Stegger, M., & Andersen, P. S. (2018). Genomic analysis reveals different mechanisms of fusidic acid resistance in Staphylococcus aureus from Danish atopic dermatitis patients. *The Journal of antimicrobial chemotherapy*, *73*(4), 856–861. https://doi.org/10.1093/jac/dkx481

ElSayed, N., Ashour, M., & Amine, A. (2018). Vancomycin resistance among *Staphylococcus aureus* isolates in a rural setting, Egypt. *Germs*, *8*(3), 134–139. https://doi.org/10.18683/germs.2018.1140

Emaneini, M., Bigverdi, R., Kalantar, D., Soroush, S., Jabalameli, F., Noorazar Khoshgnab, B., Asadollahi, P., & Taherikalani, M. (2013). Distribution of genes encoding tetracycline resistance and aminoglycoside modifying enzymes in Staphylococcus aureus strains isolated from a burn center. *Annals of burns and fire disasters*, *26*(2), 76–80.

Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., Peacock, S. J., Smith, J. M., Murphy, M., Spratt, B. G., Moore, C. E., & Day, N. P. (2003). How clonal is Staphylococcus aureus?. *Journal of bacteriology*, *185*(11), 3307–3316. https://doi.org/10.1128/jb.185.11.3307-3316.2003

Feil, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., Peacock, S. J., Smith, J. M., Murphy, M., Spratt, B. G., Moore, C. E., & Day, N. P. (2003). How clonal is Staphylococcus aureus?. *Journal of bacteriology*, *185*(11), 3307–3316. https://doi.org/10.1128/jb.185.11.3307-3316.2003

Foster T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS microbiology reviews*, *41*(3), 430–449. https://doi.org/10.1093/femsre/fux007

Fu, Z., Ma, Y., Chen, C., Guo, Y., Hu, F., Liu, Y., Xu, X., & Wang, M. (2016). Prevalence of Fosfomycin Resistance and Mutations in murA, glpT, and uhpT in Methicillin-Resistant Staphylococcus aureus Strains Isolated from Blood and Cerebrospinal Fluid Samples. *Frontiers in microbiology*, *6*, 1544. https://doi.org/10.3389/fmicb.2015.01544

Gajdács M. (2019). The Continuing Threat of Methicillin-Resistant *Staphylococcus aureus*. *Antibiotics (Basel, Switzerland)*, *8*(2), 52. https://doi.org/10.3390/antibiotics8020052

Gardete, S., & Tomasz, A. (2014). Mechanisms of vancomycin resistance in Staphylococcus aureus. *The Journal of clinical investigation*, *124*(7), 2836–2840. https://doi.org/10.1172/JCI68834

Gnanamani, A., Hariharan, P., & Paul-Satyaseela, M. (2017). Staphylococcus aureus: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach. *Frontiers In Staphylococcus Aureus*. doi: 10.5772/67338

Goering, R. (2010). Pulsed field gel electrophoresis: A review of application and interpretation in the molecular epidemiology of infectious disease. *Infection, Genetics And Evolution*, *10*(7), 866-875. doi: 10.1016/j.meegid.2010.07.023

Gómez-Sanz, E., Torres, C., Lozano, C., & Zarazaga, M. (2013). High diversity of Staphylococcus aureus and Staphylococcus pseudintermedius lineages and toxigenic traits in healthy pet-owning household members. Underestimating normal household contact?. *Comparative immunology, microbiology and infectious diseases*, *36*(1), 83–94. https://doi.org/10.1016/j.cimid.2012.10.001

Goudarzi, M., Kobayashi, N., Dadashi, M., Pantůček, R., Nasiri, M. J., Fazeli, M., Pouriran, R., Goudarzi, H., Miri, M., Amirpour, A., & Seyedjavadi, S. S. (2020). Prevalence, Genetic Diversity, and Temporary Shifts of Inducible Clindamycin Resistance *Staphylococcus aureus* Clones in Tehran, Iran: A Molecular-Epidemiological Analysis From 2013 to 2018. *Frontiers in microbiology*, *11*, 663. https://doi.org/10.3389/fmicb.2020.00663

Greenwood, D (2012). *Medical microbiology.* 12th ed. Edinburgh: Churchill Livingstone, pp.176-182

Harastani, H., Araj, G., & Tokajian, S. (2014). Molecular characteristics of Staphylococcus aureus isolated from a major hospital in Lebanon. *International Journal Of Infectious Diseases*, *19*, 33-38. doi: 10.1016/j.ijid.2013.10.007

Ida, T., Okamoto, R., Shimauchi, C., Okubo, T., Kuga, A., & Inoue, M. (2001). Identification of aminoglycoside-modifying enzymes by susceptibility testing: epidemiology of methicillin-resistant Staphylococcus aureus in Japan. *Journal of clinical microbiology*, *39*(9), 3115–3121. https://doi.org/10.1128/jcm.39.9.3115-3121.2001

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J., & Vandenesch, F. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infection and immunity*, *70*(2), 631–641. https://doi.org/10.1128/iai.70.2.631-641.2002

Javdan, S., Narimani, T., Shahini Shams Abadi, M., & Gholipour, A. (2019). Agr typing of Staphylococcus aureus species isolated from clinical samples in training hospitals of Isfahan and Shahrekord. *BMC research notes*, *12*(1), 363. https://doi.org/10.1186/s13104-019-4396-8

Jensen, S. O., & Lyon, B. R. (2009). Genetics of antimicrobial resistance in Staphylococcus aureus. *Future microbiology*, *4*(5), 565–582. https://doi.org/10.2217/fmb.09.30

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., ... McArthur, A. G. (2017). CARD 2017: Expansion and Model-Centric Curation of the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Research*, *45*(D1), D566–D573. https://doi.org/10.1093/nar/gkw1004

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Kaas, R. S., Nielsen, E. M., & Aarestrup, F. M. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. *Journal of clinical microbiology*, *52*(5), 1501–1510. https://doi.org/10.1128/JCM.03617-13

Josse, J., Laurent, F., & Diot, A. (2017). Staphylococcal Adhesion and Host Cell Invasion: Fibronectin-Binding and Other Mechanisms. *Frontiers in microbiology*, *8*, 2433. https://doi.org/10.3389/fmicb.2017.02433

Kateete, D.P., Bwanga, F., Seni, J. *et al.* CA-MRSA and HA-MRSA coexist in community and hospital settings in Uganda. *Antimicrob Resist Infect Control* 8, 94 (2019). https://doi.org/10.1186/s13756-019-0551-1

Khalil, W., Hashwa, F., Shihabi, A., & Tokajian, S. (2012). Methicillin-resistant Staphylococcus aureus ST80-IV clone in children from Jordan. Diagnostic microbiology and infectious disease, 73(3), 228-230.

Lakhundi, S., & Zhang, K. (2018). Methicillin-ResistantStaphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. *Clinical Microbiology Reviews*, *31*(4). doi: 10.1128/cmr.00020-18

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., ... Lund, O. (2012). Multilocus Sequence Typing of Total-Genome- Sequenced Bacteria. *Journal of Clinical Microbiology*, *50*(4), 1355–1361. https://doi.org/10.1128/JCM.06094-11

Lee, A. S., de Lencastre, H., Garau, J., Kluytmans, J., Malhotra-Kumar, S., Peschel, A., & Harbarth, S. (2018). Methicillin-resistant Staphylococcus aureus. *Nature reviews. Disease primers*, *4*, 18033. https://doi.org/10.1038/nrdp.2018.33

Lina, G., Piemont, Y., Godail-Gamot, F., Bes, M., Peter, M., & Gauduchon, V. et al. (1999). Involvement of Panton-Valentine Leukocidin--Producing Staphylococcus aureus in Primary Skin Infections and Pneumonia. *Clinical Infectious Diseases*, *29*(5), 1128-1132. doi: 10.1086/313461

Lister, J. L., & Horswill, A. R. (2014). Staphylococcus aureus biofilms: recent developments in biofilm dispersal. *Frontiers in cellular and infection microbiology*, *4*, 178. https://doi.org/10.3389/fcimb.2014.00178

Liu, Y., & Ji, Y. (2020). Multilocus Sequence Typing of Staphylococcus aureus. *Methods in molecular biology (Clifton, N.J.)*, *2069*, 95–102. https://doi.org/10.1007/978-1-4939-9849-4_7

Livermore D. M. (2000). Antibiotic resistance in staphylococci. *International journal of antimicrobial agents*, *16 Suppl 1*, S3–S10. https://doi.org/10.1016/s0924-8579(00)00299-5

Lowy F. D. (2003). Antimicrobial resistance: the example of Staphylococcus aureus. *The Journal of clinical investigation*, *111*(9), 1265–1273. https://doi.org/10.1172/JCI18535

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, *18*(3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x

Mairi, A., Touati, A., & Lavigne, J. P. (2020). Methicillin-Resistant *Staphylococcus aureus* ST80 Clone: A Systematic Review. *Toxins*, *12*(2), 119. https://doi.org/10.3390/toxins12020119

Malachowa, N., & DeLeo, F. R. (2010). Mobile genetic elements of Staphylococcus aureus. *Cellular and molecular life sciences : CMLS*, *67*(18), 3057–3071. https://doi.org/10.1007/s00018-010-0389-4

McClure, J. A., Conly, J. M., Lau, V., Elsayed, S., Louie, T., Hutchins, W., & Zhang, K. (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. *Journal of clinical microbiology*, *44*(3), 1141–1144. https://doi.org/10.1128/JCM.44.3.1141-1144.2006

McGuinness, W. A., Malachowa, N., & DeLeo, F. R. (2017). Vancomycin Resistance in *Staphylococcus aureus*. *The Yale journal of biology and medicine*, *90*(2), 269–281.

Mellmann, A., Weniger, T., Berssenbrügge, C., Keckevoet, U., Friedrich, A. W., Harmsen, D., & Grundmann, H. (2008). Characterization of clonal relatedness among the natural population of Staphylococcus aureus strains by using spa sequence typing and the BURP (based upon repeat patterns) algorithm. *Journal of clinical microbiology*, *46*(8), 2805–2808. https://doi.org/10.1128/JCM.00071-08

Moormeier, D. E., & Bayles, K. W. (2017). Staphylococcus aureus biofilm: a complex developmental organism. *Molecular microbiology*, *104*(3), 365–376. https://doi.org/10.1111/mmi.13634

Namvar, A. E., Bastarahang, S., Abbasi, N., Ghehi, G. S., Farhadbakhtiarian, S., Arezi, P., Hosseini, M., Baravati, S. Z., Jokar, Z., & Chermahin, S. G. (2014). Clinical characteristics of Staphylococcus epidermidis: a systematic review. *GMS hygiene and infection control*, *9*(3), Doc23. https://doi.org/10.3205/dgkh000243

Neoh, H. M., Tan, X. E., Sapri, H. F., & Tan, T. L. (2019). Pulsed-field gel electrophoresis (PFGE): A review of the "gold standard" for bacteria typing and current alternatives. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*, *74*, 103935. https://doi.org/10.1016/j.meegid.2019.103935

O'Brien, L., Kerrigan, S. W., Kaw, G., Hogan, M., Penadés, J., Litt, D., Fitzgerald, D. J., Foster, T. J., & Cox, D. (2002). Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Molecular microbiology*, *44*(4), 1033–1044. https://doi.org/10.1046/j.1365-2958.2002.02935.x

Oksuz, L., Dupieux, C., Tristan, A., Bes, M., Etienne, J., & Gurler, N. (2013). The high diversity of MRSA clones detected in a university hospital in istanbul. *International journal of medical sciences*, *10*(12), 1740–1745. https://doi.org/10.7150/ijms.6438

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., ... Stevens, R. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research*, *42*(Database issue), D206-214. https://doi.org/10.1093/nar/gkt1226

Peacock, S. J., & Paterson, G. K. (2015). Mechanisms of Methicillin Resistance in Staphylococcus aureus. *Annual review of biochemistry*, *84*, 577–601. https://doi.org/10.1146/annurev-biochem-060614-034516

Plata, K., Rosato, A. E., & Wegrzyn, G. (2009). Staphylococcus aureus as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta biochimica Polonica*, *56*(4), 597–612.

R. G. Naffa, S. M. Bdour, H. M. Migdadi, and A. A.Shehabi, "Enterotoxicity and genetic variation among clinical Staphylococcus aureusisolates in Jordan," Journal of Medical Microbiology , vol. 55, no. 2, pp. 183–187, 2006

Sato'o, Y., Omoe, K., Naito, I., Ono, H. K., Nakane, A., Sugai, M., Yamagishi, N., & Hu, D. L. (2014). Molecular epidemiology and identification of a Staphylococcus aureus clone causing food poisoning outbreaks in Japan. *Journal of clinical microbiology*, *52*(7), 2637–2640. https://doi.org/10.1128/JCM.00661-14

Saunders, N. A., & Holmes, A. (2007). Multilocus sequence typing (MLST) of Staphylococcus aureus. *Methods in molecular biology (Clifton, N.J.)*, *391*, 71–85. https://doi.org/10.1007/978-1-59745-468-1_6

Schmitz, F. J., Steiert, M., Tichy, H. V., Hofmann, B., Verhoef, J., Heinz, H. P., Köhrer, K., & Jones, M. E. (1998). Typing of methicillin-resistant Staphylococcus aureus isolates from Düsseldorf by six genotypic methods. *Journal of medical microbiology*, *47*(4), 341–351. https://doi.org/10.1099/00222615-47-4-341

Schwartz, D. C., & Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, *37*(1), 67–75. https://doi.org/10.1016/0092-8674(84)90301-5

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Seyedi-Marghaki, F., Kalantar-Neyestanaki, D., Saffari, F., Hosseini-Nave, H., & Moradi, M. (2019). Distribution of Aminoglycoside-Modifying Enzymes and Molecular Analysis of the Coagulase Gene in Clinical Isolates of Methicillin-Resistant and Methicillin-Susceptible Staphylococcus aureus. *Microbial drug resistance (Larchmont, N.Y.)*, *25*(1), 47–53. https://doi.org/10.1089/mdr.2017.0121

Sonnevend, Á., Blair, I., Alkaabi, M., Jumaa, P., Al Haj, M., Ghazawi, A., Akawi, N., Jouhar, F. S., Hamadeh, M. B., & Pál, T. (2012). Change in meticillin-resistant Staphylococcus aureus clones at a tertiary care hospital in the United Arab Emirates over a 5-year period. *Journal of clinical pathology*, *65*(2), 178–182. https://doi.org/10.1136/jclinpath-2011-200436

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica*, *115*(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

Tan, L., Li, S. R., Jiang, B., Hu, X. M., & Li, S. (2018). Therapeutic Targeting of the *Staphylococcus aureus* Accessory Gene Regulator (*agr*) System. *Frontiers in microbiology*, *9*, 55. https://doi.org/10.3389/fmicb.2018.00055

Tang, Y. W., Waddington, M. G., Smith, D. H., Manahan, J. M., Kohner, P. C., Highsmith, L. M., Li, H., Cockerill, F. R., 3rd, Thompson, R. L., Montgomery, S. O., & Persing, D. H. (2000). Comparison of protein A gene sequencing with pulsed-field gel electrophoresis and epidemiologic data for molecular typing of methicillin-resistant Staphylococcus aureus. *Journal of clinical microbiology*, *38*(4), 1347–1351.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of clinical microbiology*, *33*(9), 2233–2239.

Thompson, T., & Brown, P. D. (2014). Comparison of antibiotic resistance, virulence gene profiles, and pathogenicity of methicillin-resistant and methicillin-susceptible Staphylococcus aureus using a Caenorhabditis elegans infection model. *Pathogens and global health*, *108*(6), 283–291. https://doi.org/10.1179/2047773214Y .0000000155

Tokajian, S., Haddad, D., Andraos, R., Hashwa, F., & Araj, G. (2011). Toxins and Antibiotic Resistance in Staphylococcus aureus Isolated from a Major Hospital in Lebanon. *ISRN microbiology*, *2011*, 812049. https://doi.org/10.5402/2011/812049

TOKAJIAN, S., KHALIL, P., JABBOUR, D., RIZK, M., FARAH, M., HASHWA, F., & ARAJ, G. (2010). Molecular characterization of Staphylococcus aureus in Lebanon. *Epidemiology And Infection*, *138*(5), 707-712. doi: 10.1017/s0950268810000440

Turner, N.A., Sharma-Kuinkel, B.K., Maskarinec, S.A. *et al.* Methicillin- resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nat Rev Microbiol* 17, 203–218 (2019). https://doi.org/10.1038/s41579-018- 0147-4

Udo, E. E., Al-Lawati, B. A., Al-Muharmi, Z., & Thukral, S. S. (2014). Genotyping of methicillin-resistant Staphylococcus aureus in the Sultan Qaboos University Hospital, Oman reveals the dominance of Panton-Valentine leucocidin-negative ST6-IV/t304 clone. *New microbes and new infections*, *2*(4), 100–105. https://doi.org/10.1002/nmi2.47

Udo, E. E., Boswihi, S. S., & Al-Sweih, N. (2016). High prevalence of toxic shock syndrome toxin-producing epidemic methicillin-resistant Staphylococcus aureus 15 (EMRSA-15) strains in Kuwait hospitals. *New microbes and new infections*, *12*, 24–30. https://doi.org/10.1016/j.nmni.2016.03.008

van Hal, S. J., Jensen, S. O., Vaska, V. L., Espedido, B. A., Paterson, D. L., & Gosbell, I. B. (2012). Predictors of mortality in Staphylococcus aureus Bacteremia. (2), 362–386. https://doi.org/10.1128/CMR.05022-11

Wang, T., Tanaka, M., & Sato, K. (1998). Detection of grlA and gyrA mutations in 344 Staphylococcus aureus strains. *Antimicrobial agents and chemotherapy*, *42*(2), 236–240.

Wielders, C. L., Fluit, A. C., Brisse, S., Verhoef, J., & Schmitz, F. J. (2002). mecA gene is widely disseminated in Staphylococcus aureus population. *Journal of clinical microbiology*, *40*(11), 3970–3975. https://doi.org/10.1128/jcm.40.11.3970-3975.2002

Wu, D., Chen, Y., Sun, L., Qu, T., Wang, H., & Yu, Y. (2018). Prevalence of Fosfomycin Resistance in Methicillin-Resistant Staphylococcus aureus Isolated from Patients in a University Hospital in China from 2013 to 2015. *Japanese journal of infectious diseases*, *71*(4), 312–314. https://doi.org/10.7883/yoken.JJID.2018.013

Xu, S. X., & McCormick, J. K. (2012). Staphylococcal superantigens in colonization and disease. *Frontiers in cellular and infection microbiology*, *2*, 52. https://doi.org/10.3389/fcimb.2012.00052

Yadegar, A., Sattari, M., Mozafari, N., & Goudarzi, G. (2009). Prevalence of the Genes Encoding Aminoglycoside-Modifying Enzymes and Methicillin Resistance Among Clinical Isolates ofStaphylococcus aureusin Tehran, Iran. *Microbial Drug Resistance*, *15*(2), 109-113. doi: 10.1089/mdr.2009.0897

Yang, J. A., Park, D. W., Sohn, J. W., & Kim, M. J. (2006). Novel PCR-restriction fragment length polymorphism analysis for rapid typing of staphylococcal cassette chromosome mec elements. *Journal of clinical microbiology*, *44*(1), 236–238. https://doi.org/10.1128/JCM.44.1.236-238.2006

Yarwood, J. M., Bartels, D. J., Volper, E. M., & Greenberg, E. P. (2004). Quorum sensing in Staphylococcus aureus biofilms. *Journal of bacteriology*, *186*(6), 1838–1850. https://doi.org/10.1128/jb.186.6.1838-1850.2004

Yoon, E. J., Lee, H., Kim, D., Shin, J. H., Shin, J. H., & Jeong, S. H. (2019). Methicillin-Resistant *Staphylococcus aureus* Blood Isolates Harboring a Novel Pseudo-staphylococcal Cassette Chromosome *mec* Element. *Frontiers in microbiology*, *10*, 540. https://doi.org/10.3389/fmicb.2019.00540

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M., & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *The Journal of antimicrobial chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M., & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *The Journal of antimicrobial chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zhang, K., McClure, J. A., Elsayed, S., Louie, T., & Conly, J. M. (2005). Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. *Journal of clinical microbiology*, *43*(10), 5026–5033. https://doi.org/10.1128/JCM.43.10.5026-5033.2005

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., & Wishart, D. S. (2011). PHAST: A Fast Phage Search Tool. *Nucleic Acids Research*, *39*(Web Server issue), W347–W352. https://doi.org/10.1093/nar/gkr485