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Genetic Diversity of *Staphylococcus aureus* in Lebanon

By

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Genetic Diversity of *Staphylococcus aureus* in Lebanon

Houda Haitham Harastani

Abstract

Staphylococcus aureus, one of the most significant human pathogens, causes different infections ranging in severity from mild superficial skin infections to life threatening bacteremia and endocarditis. The occurrence and dissemination of methicillin-resistant variants (MRSAs) in clinical settings has raised the concern for the constant increase of nosocomial infections all over the world. Additionally, the appearance of MRSA among the community (community acquired [CA-MRSA]) and its risk of being introduced into hospitals is a matter of great concern. Effective epidemiological studies based on typing are indispensible for confirming the genetic relatedness of closely related isolates belonging to the same clonal lineages as well as for screening and controlling the occurrence and spread of epidemic clones in the region. This study aimed at the molecular characterization of *S. aureus* in Lebanon. 132 *S. aureus* isolates, 39 MRSA and 93 Methicillin-susceptible (MSSA), were collected from the American University of Beirut Medical Center (AUB-MC) and were characterized using different typing methods including *S. aureus* protein A (*spa*) typing, staphylococcal chromosomal cassette (SCC*mec*) typing of MRSA, multilocus sequence typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE). The detection of Panton-Valentine Leukocidin (PVL) and the distribution of SCC*mec* types I-VIII were performed by multiplex PCR assays. PVL were detected in 54% of MRSA and 12% of MSSA. Seventy one different *spa* types were identified and clustered into 40 groups with the most common *spa* type being t044 (13%) among MRSA and t021 (8%) among MSSA. SCC*mec* typing showed the prevalence of types IV (d, e, f, g, and h) (49%) followed by IVa (31%), V (13%), IVc (5%) and III (3%). Twenty five allelic profiles or STs were identified by MLST with the major STs being ST80 (11%) and ST30 (8%). PFGE revealed 32 groups by applying 80% as a cutoff similarity. Clustering *mec* typing

with MLST revealed that ST80-MRSA-IV and ST30-MSSA were the predominant clones in Lebanon. The present study showed the great diversity between MRSA and MSSA isolates in Lebanon compared to previous conducted studies. Systematic surveillance is required for isolates associated with both hospital- and community-acquired infections to limit and control the spread of *S. aureus*. Future studies with larger number of samples from different countries are essential in order to better assess and characterize MRSA and MSSA in the region. Determining the prevailing genetic populations in Lebanon along with their prevalence in causing diseases will help in developing measures to control the spread of these potentially serious infections.

Keywords: *Staphylococcus aureus*, MRSA, MSSA, MLST, *spa* typing, PFGE, SCC*mec* typing.

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GLOSSARY

bp: Base pair

BURP: Based upon repeat pattern

CA-MRSA: Community-acquired methicillin-resistant *Staphylococcus aureus*

CC: Clonal complex

CHEF: Contour-clamped homogenous electric field

CRA: Congo red agar

eDNA: Extracellular DNA

EPS: Extracellular polymeric substances

HA-MRSA: Hospital-acquired methicillin-resistant *Staphylococcus aureus*

MDR: Multi-drug resistance

MLST: Multi-locus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSA: Mannitol salt agar

MSCRAMMS: Microbial surface components recognizing adhesive matrix molecules

MSSA: Methicillin-sensitive *Staphylococcus aureus*

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

PIA: Polysaccharide intercellular adhesin

PNAG: Poly-N-acetylglucosamine

PVL: Panton-Valentine Leukocidin

RFLPs: Restriction fragment length polymorphisms

SAgs: Superantigen toxins

S. aureus: Staphylococcus aureus

SCC: Staphylococcal chromosomal cassette

SE: Staphylococcal enterotoxin

SLV: Single locus variant

spa-CC: *spa* clonal complex

spa: Gene for Staphylococcal Protein A

SSTI: Skin and soft tissue infection

ST: Sequence type

TAE: Tris-Acetic Acid EDTA

TBE: Tris-Boric Acid EDTA

TSA: Tryptone soy agar

TSS: Toxic shock syndrome

TSST-1: Toxic shock syndrome toxin 1

VISA: Vancomycin-intermediate *S. aureus*

VRSA: Vancomycin-resistant *S. aureus*

CHAPTER ONE

INTRODUCTION

Staphylococcus aureus, a highly adaptive and versatile gram-positive bacterium, is considered one of the most commonly isolated human pathogens (McCarthy and Lindsay 2010; David and Daum 2010). It colonizes asymptomatically the nasal mucosa of around 30% of humans and is responsible for a variety of diseases ranging in severity from mild superficial skin infections to life threatening deep-seated infections (Yamamoto et al. 2010). It is an important pathogen that has gained interest in the healthcare as well as in community settings (Chung et al. 2011). Pathogenesis of *S. aureus* is attributable to a variety of expressed cell-surface as well as secreted virulence factors which include: exfoliative toxins, enterotoxins, exotoxins, protein A, catalase and others (Xie et al. 2011). Implication of biofilms during infection constitutes an additional virulence factor that helps the bacterium persist harsh environments within the host and escape the immune system (Dhanawade et al. 2010).

Epidemiology of *S. aureus* has been greatly influenced by the prevalence and fast expansion of drug-resistant strains which has complicated traditional treatment regimens (Xie et al. 2011; Shittu et al. 2011). Soon after the introduction of methicillin for the treatment of penicillin-resistant strains, the first methicillin-resistant *S. aureus* (MRSA) strain was isolated (Blanc et al. 2007). These strains conferred resistance not only to methicillin but also to antibiotics within the β-lactam family and antibiotics from other families as well (Blanc et al. 2007). Since then, MRSA strains became increasingly endemic and were prototypes of nosocomial infections and thus collectively known as hospital-acquired MRSAs (HA-MRSAs) (Yamamoto et al. 2010; Blanc et al. 2007). Later, MRSA strains have shown a capacity for causing outbreaks in the community especially in the young and previously un-hospitalized patients suggesting a major epidemiological change (Beaume et al. 2010). CA-MRSA became then endemic

pathogens with specific clones confined to certain geographical areas (Chambers and DeLeo 2009). The increase use of vancomycin as a treatment of choice for the ever growing burden of MRSA infections has led to emergence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) strains which has limited the clinical reliance on vancomycin against invasive MRSA infections (Chambers & DeLeo 2009; David & Daum 2010).

Confronted with these epidemiological changes, knowledge about epidemiologically prevalent circulating MRSA clones can help in developing effective strategies that aid in controlling spread, optimizing treatment and determining mode of pathogenicity (Beaume et al. 2010; Dauwalder et al. 2008). Monitoring the evolutionary process of prevalent MRSA clones through current genotyping techniques is a crucial step to reveal genetic clonality and relatedness among isolates. Among the most valuable typing techniques currently available for understanding pathogen evolution are: *spa* typing, SCC*mec* typing, MLST and PFGE.

In this study, a collection of 132 *S. aureus* isolates from the AUB-MC collected between May-October 2011 were genotyped by PFGE, MLST, *spa* and SCC*mec*. Objectives of this study were to (i) investigate clonal structure of MSSA and MRSA strains collected from AUB-MC, which include a component of expatriate population from different countries that help in providing consequent opportunity for dissemination of international clones, (ii) explore the relationship between current and previously characterized clones in Lebanon as well as those prevalent in the region, (iii) identify the risk factors for MRSA carriage among the Lebanese population and (iv) determine the antibiotic susceptibility profiles of MSSA and MRSA isolates.

(Part of this work will be presented at the 112th American society for microbiology general meeting held in San Francisco, CA, June 2012).

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of *Staphylococcus aureus*

Staphylococcus aureus, a Gram-positive ubiquitous bacterium, is considered the causative agent of a broad spectrum of diseases ranging in severity from mild localized skin infections to life threatening diseases (Beaume et al. 2010). *S. aureus* is a normal flora that colonizes asymptomatically the nasal passages in approximately 30% of normal people (Beaume et al. 2010; Lamers et al. 2011).

S. aureus is a spherical, non-motile and non-spore forming bacterium (Tolan et al. 2010) that occur microscopically as grape-like clusters while forming large golden to yellow colonies when cultivated on rich media (Todar 2011). Taxonomically, genus *S. aureus* belongs to the *Staphylococcaceae* family that also includes three less encountered genera: *Gamella*, *Macrococcus* and *Salinicoccus*. The bacterium is a facultative anaerobe and has the ability to regulate its metabolism to withstand drastic changes in environmental conditions (Beaume et al. 2010; Todar 2011). *S. aureus* can tolerate high salt concentrations up to 15% NaCl and grow at a temperature that ranges from 15˚C to 45˚C. It is distinguished by its ability to produce catalase, an enzyme that hydrolyzes hydrogen peroxide into water and oxygen gas, and being oxidase negative (Todar 2011).

S. aureus establishes and causes disease through the production of surface-associated as well as secreted virulence factors (Fey et al. 2003). The ability of *S. aureus* to form biofilms is an additional virulence factor that helps it to escape antimicrobial chemotherapies and to resist host responses (Croes et al. 2009).

Treatment of *S. aureus* in the era of resistance against multi-antimicrobial drugs is sometimes associated with failure and increase of disease severity (Graveland et al.

2010). Mechanism of resistance is mainly associated with horizontal gene transfer and mutations (Chambers and DeLeo 2009). Despite being originally restricted to be a hospital-associated pathogen, MRSA has gained an increased dissemination among community settings as well, suggesting an epidemiological change (Tavares et al. 2010; Lamaro-Cardoso et al. 2009).

2.2 Occurrence

Epidemiology of *S. aureus* infections has been influenced greatly by the rapid emergence of antibiotic resistance (Chambers and DeLeo 2009). Mortality rates due to *S. aureus* infections prior to the introduction of penicillin for medical use in the 1940s were approximated to be around 80% (Skinner and Keefer 1941). In 1942, two years after the use of penicillin, the first penicillin-resistant *S. aureus* strain was isolated in a hospital and by the early 1950s and 1960s, penicillin-resistant strains became pandemic in the community (Chambers and DeLeo 2009; Deurenberg and Stobberingh 2008). Infections caused by the time were due to *S. aureus* clone known as phage-type 80/81 that has been successfully eradicated after the introduction of methicillin in 1960 (Chambers and DeLeo 2009). Again, soon *S. aureus* has gained resistance to methicillin in the first report of isolation in a hospital in UK in 1961 (David and Daum 2010). Resistance against methicillin, which was identified more than 20 years later, was due to the *mecA* gene that codes for a low affinity penicillin-binding protein (PBP2a). The mechanism of resistance was different from that against penicillin since no drug-inactivation was observed (Chambers and DeLeo 2009).

Today, MRSA is becoming a major public health threat with some clones predominating specific geographical areas, while others achieving a pandemic spread (Monecke et al. 2011). The increased biological diversity of MRSA has enabled exploitation of the small and relatively controllable ecological niches outside hospital settings, which has increased the costs of infection prevention and control measures worldwide (Monecke et al. 2011). CA-MRSA is becoming well adapted to the community and is causing unexpected cases of diseases. Therefore, new

countermeasures against the evolving threat should be considered and designed (Yamamoto et al. 2010).

2.3 Pathogenesis of *Staphylococcus aureus*

Illness caused by *S. aureus* is attributable to a variety of cell surface and secreted virulence factors that elicit host immune responses (Argudin et al. 2009). These include proteins, adhesins, toxins, hemolysins, superantigens and leukocidins (Chi et al. 2010; Haveri et al. 2008).

Following introduction into the host, *S. aureus*' surface proteins including fibrongen-, fibronectin- and collagen-binding proteins recognizes host matrices and promote bacterial colonization and adherence constituting initial stages of infection (Haveri et al. 2008). Upon successful adherence, the bacterium seeks survival by obtaining nutrients and dissemination through the production of a variety of enzymes and toxins responsible for the pathological events observed during infection development. These include hemolysins (α, β, γ, and δ) and leukocidins such as Panton-Valentine Leukocidin (PVL) that promote host cell lysis. PVL, which is a prophage-encoded bi-component pore-forming protein, is associated mainly with CA-MRSA strains. It is encoded by two genes: *lukS-PV* and *lukF-PV* residing in genomes of some bacteriophages (e.g.: ΦSa2958, ΦSa2MW, ΦPVL) and these are readily transferrable following selective bacterial infection (Boakes et al. 2011). At elevated concentrations, PVL causes host cell lysis; however at lower concentrations, PVL primes neutrophils to release inflammatory mediators such as leukotriene B4, IL-8, granule contents and reactive oxygen species (Chambers and DeLeo 2009). Although its role in pathogenicity remains controversial, many murine-conducted studies show the role of PVL in mitochondrial inactivation and apoptosis as well as its association with certain established diseases such as necrotizing pneumonia and skin and soft tissue infections (SSTIs) (David and Daum 2010).

Burden of *S. aureus* is associated also with production of superantigen toxins (SAgs) that stimulates host's defenses leading to lethal consequences in the course of

rapid progression of infection (Haveri et al. 2008). Pyrogenic toxins such as toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxins (SEs) are responsible for establishing toxic shock syndrome (TSS) and other related infections through T-cell and macrophage activation causing massive, uncontrollable release of pro-inflammatory cytokines (Argudin et al. 2009; Fey et al. 2003).

Exofoliative toxins (ETA, ETB and ETD) are serine proteases that are capable of digesting desmoglein 1 in stratum granulosum resulting in an exfoliated epidermis. Both ETA and ETB cause staphylococcal scalded skin syndrome (SSSS) in infants and bullous impetigo in children, while ETD not only cause bullous impetigo but also deep pyoderma in adults (Nishifuji et al. 2008).

Additionally, protein A which is a cell-surface associated protein represents an important virulence factor for *S. aureus*. It has the ability to bind the Fc portion of immunoglobulin G (IgG) by that inhibiting opsonization and subsequent phagocytosis. It is also associated with initiating a cascade of pro-inflammatory responses in human airway epithelial cells through activating tumor necrosis factor receptor 1, B cells and other lignads (David and Daum 2010).

2.4 Biofilm formation by *Staphylococcus aureus*

Pathogenesis of *S. aureus* is also attributable to the formation of biofilms which help the bacterium persist and withstand hostile environments within the host (Dhanawade et al. 2010). Biofilms are not mere aggregations of bacteria accumulated on a surface, but rather are large, complex and organized communities that allow inter-bacterial interactions and serve as transportation networks (Goodman et al. 2011). Biofilm formation is a multi-step process initiated by first adhering to a biotic or abiotic surface with the aid of specific adhesins, collectively known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMS). Following attachment, bacteria stick to each other and produce extracellular polymeric substances (EPS) with or without the incorporation of host components to develop a mature biofilm (Croes et al. 2009). EPS accounts for 90% of the actual dry mass of the biofilm and

creates a semi-permeable barrier that not only control entry of essential metabolites but also protects resident bacteria against effectors of the immune system as well as applied therapeutic regimens (Goodman et al. 2011). A major component in constructing biofilms is the polysaccharide intercellular adhesin (PIA), also referred to as poly-N-acetylglucosamine (PNAG), a β-1,6-N-acetylglucosamine polymer, synthesized by N-acetylglucoamyl transferase encoded by the *A* gene in the *ica* operon. *ica* operon contains also a regulatory element of three other genes (*B*, *C* and *D*) along with a transposable element, *IS*256, which controls expression of the *icaADBC* genes by excision or insertion at different locations on the operon (Diemond-Hernández et al. 2010). Co-expression of the *icaD* along with *icaA* is thought to increase the activity of the transferase enzyme. *icaB* gene product is in charge of deacetylating growing PIA, while *icaC* product is responsible for externalization and elongation of the maturing polysaccharide (Diemond-Hernández et al. 2011). Biofilm formation however is not restricted to the production of PNAG, since isolation of PNAG-deficient strains where found to exhibit strong biofilm ability. The presence of extracellular DNA (eDNA) as well as proteinaceous adhesins may contribute to the establishment of biofilms (Izano et al. 2008). As it has been shown that regulatory pathways for biofilm formation varies among strains, it is important to examine if these differences can also be linked to differentness in clonal lineages (Croes et al. 2009).

2.5 Resistance to methicillin

In 1961, shortly after the introduction of methicillin into clinical practice against penicillin-resistant *S. aureus* strains, isolation of the first methicillin-resistant strain was reported in the United Kingdom (David and Daum 2010). Compared to pencillinase-mediated that has a narrow resistance spectrum, methicillin-resistant strains are accompanied with a broad β-lactam antibiotic class resistance and are frequently resistant to antibiotics from other classes (Chambers and DeLeo 2009; Blanc et al. 2007). MRSA is considered among the most prevalent antibiotic-resistant pathogen in hospital settings (Mollema et al. 2010) but has clearly showed the ability for causing outbreaks in the community suggesting a major epidemiological evolution (Beaume et

al. 2010). MRSA carriage among asymptomatic people is considered a prerequisite for most MRSA infections and aid in the spreading of the organism within health care as well as community settings (Mollema et al. 2010). Compared to hospital-acquired MRSA (HA-MRSA) and MSSA, infections caused by CA-MRSA are considered the most lethal and tend to have worst clinical outcomes (Chambers and DeLeo 2009).

Expression of methicillin resistance is mediated by the *mecA* gene that codes for the penicillin binding protein PBP2a, a protein with low affinity to β-lactam antibiotics (Ghaznavani-Rad et al. 2010). The *mecA* gene resides on a heterogeneous genetic mobile element carried by a staphylococcal chromosome known as staphylococcal cassette chromosome *mec* (SCC*mec*) elements (Kader et al. 2011). SCC*mec* elements share common features and these include: (i) integration in the *orfX* gene at a specific bacterial chromosomal attachment site (*attB*SCC) through recognition of a specific nucleotide sequence known as the integration site sequence of SCC (ISS) (Chen et al. 2010); (ii) flanking repeated nucleotide and inverted complementary sequences (Kader et al. 2011); (iii) presence of two essential genetic elements: the *mec* gene complex (central of methicillin resistance), and the cassette chromosome recomibnase (*ccr*) gene complex (accounting for the motility of SCC*mec* element and surrounding sequences) (Kondo et al. 2007; Chen et al. 2010). In *S. aureus*, three major *mec* complexes have been identified known as classes A-C of which three major allotypes (*ccr*AB1- *ccr*AB3) with one sporadic allotype (*ccr*AB4) exists and a newly identified *ccr* gene complex containing only one gene (*ccr*C) (Kader et al. 2011). SCC*mec* elements can be further differentiated based upon three "junkyard" (J) regions: J1 region: between the *ccr* gene complex and the right flanking chromosomal region, J2 region: joining *ccr* gene complex to the *mec* gene complex, and J3 region: region between the *ccr* gene complex and the left flanking chromosomal region (Kondo et al. 2007). To date, at least eight major SCC*mec* types have been identified I-VIII based on the combination of class *mecA* gene complex and the type of the *ccr* gene complex (Smyth et al. 2011) while variations in the J regions are utilized to define SCC*mec* subtypes (Ishihara et al. 2010).

2.6 Molecular typing of *Staphylococcus aureus*

The prevalence and rapid dissemination of MRSA after introduction of methicillin into clinical use had paved the way for the appearance of hospital multi-resistant clones, an increasingly growing concern as it is a leader cause for nosocomial infections all over the world (Strommenger et al. 2008). Recently, the emergence of CA-MRSA and its potential risk for introduction into hospitals is a threat to the public health (Ghaznavani-Rad et al. 2010). For that, knowledge about epidemiologically prevalent MRSA clones is indispensable to limit their spread, determine their mode of pathogenicity as well as to optimize their treatment (Dauwalder et al. 2008). Numerous typing methods are currently available to assess strain relatedness in terms of global evolution and genetic clonality, the key for monitoring the evolutionary process responsible for the emergence and the geographical spread of clones (Hall et al. 2009; Dauwalder et al. 2008). Thus it's important to have access to specific and precise typing methods that offer cost-effective intervention and prevention strategies (Conceiçáo et al. 2009). It is noteworthy also that typing techniques must not only be accurate but also time-limiting for the continous survellinace and outbreak investigations of pandameic clones (Church et al. 2011). The current avaliable methods used for typing *S. aureus* include *Sma*I macrorestriction using pulsed-field gel electrophoresis (PFGE), sequencing of seven houskeeping genes with multi-locus sequence typing (MLST), sequencing of protein A (*spa* typing) and SCC*mec* typing, the key for defning MRSA type. Each of these methods offer advantages and disadvantages regarding typeability, reproducibility, rapidity, discriminatory power, ease of use and interpretation as well as cost (Singh et al. 2006).

2.6.1 Pulsed-field gel electrophoresis (PFGE)

PFGE using *Sma*I is considered the "gold standard" for typing MRSA isolates (Church et al. 2011; Bosch et al. 2010; Faria et al. 2008; Malachowa et al. 2005). This technique, which was developed by Schwarz and Cantor (1984), involves growing the organism in a broth or solid media combined with low melting agarose forming plugs containing the whole bacteria. Embedded organisms are then exposed to in situ lysis and

digestion using an infrequently cutting restriction endonuclease (Olive and Bean 1999). *Sma*I (5'-CCC^GGG-3') hexanucleotide recognition sequence rarely cuts the A + T rich gram positive bacteria, thus it has been shown to have the highest performance for *S. aureus* (Goering 2010). The resulting series of fragments is of different sizing patterns and are referred to restriction fragment length polymorphisms (RFLPs) (Singh et al. 2006). These fragments are, however, too large to be separated by conventional electrophoresis. Thus, alternative methods, generally defined as PFGE, rely on time-associated size-dependent reorientation of DNA migration by switching the direction of an electrical current in a predetermined pattern (Goering 2010). One common approach utilized by PFGE is contour-clamped homogenous electric field (CHEF) that contains multiple electrodes for switching electric field at 120˚ angles ensuring efficient separation (Singh et al. 2006). In order to interpret the resulting DNA fragment patterns and transform them into potentially meaningful information in an epidemiological text, guidelines proposed by Tenover et al. (1995) involves comparison of bacterial isolates to obtain their genomic relatedness (Goering 2010). Accordingly, banding patterns that differ in three fragments is probably the result of a single genetic event and thus isolates are classified as epidemiologically related subtypes of the same strain, while isolates that differs in the position of more than three restriction fragments are considered to be unrelated (Singh et al. 2006). Today, softwares are accessible for analyzing DNA fingerprint data as well as creating databases by storing banding patterns for a series of gels. The resulting phylogenic output is referred to as a dendrogram, a graphical representation of genetic similarities and differences between groups (Singh et al. 2006). Although PFGE is highly reproducible and stays an important tool in micro- and macro-epidemiological surveys, the technique suffers from several disadvantages. These include: high cost in outbreaks, technical challenge, time consumption, labor intensiveness, complex inter-laboratory exchange of results, as well as hampered typeability of isolates having methylated S*ma*I sites (Church et al. 2011; Bosch et al. 2010; Georing 2010; Hallin et al. 2007).

2.6.2 Multi-locus sequence typing (MLST)

Accurate determination of genetic relatedness among bacterial isolates constitutes the basics of epidemiological and evolutionary studies. Recent nucleotide-sequencing methodologies at multiple housekeeping loci is increasing and offers advantages in inferring strain relatedness as well as reconstructing evolutionary events (Feil et al. 2004). Characterization of *S. aureus* isolates have been determined by scanning sequences of approximately 450 bp internal fragments of seven housekeeping genes using MLST. The technique which was developed by Enright et al. in 2000, offers high discriminatory power in indexing slowly accumulated variations that serves in long-term global epidemiological studies (Enright et al. 2002). The seven housekeeping genes are: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (Deurenberg and Stobberingh 2008). The combination of alleles at the seven loci is defined as an allelic profile which is assigned a unique sequence type (ST). Studying the evolutionary events between isolates using MLST reveals that a significant proportion of isolates belong to a confined number of clusters that share related genotypes, commonly referred to as clonal complexes (CCs) (Feil et al. 2004). Implementation of algorithms such as the based upon related sequence type (eBURST) algorithm (www.eburst.mlst.net) to cluster isolates into CCs is based on determining a similarity threshold for group definition such as applying the stringent method with defining six out of seven housekeeping genes as identical and thus clustering isolates into a single CC (Deurenberg and Stobberingh 2008). Clonal complexes are thus composed of a prevalent genotype among less common relatives of this genotype where the putative ancestor of the CC is the ST with the largest number of single locus variants (SLVs) (Deurenberg and Stobberingh 2008; Feil et al. 2004). MLST is proven to be a useful technique for molecular evolution studies due to ease of performance as well as providing fast, unambiguous and portable data that are exchanged easily due to the presence of available public databases (Conceiçáo et al. 2009; Hallin et al. 2007). However, still MLST could not be useful in local outbreaks due to its high cost, labor intesivness and time consumption

(Strommenger et al. 2006; Deurenberg and Stobberingh 2008). Combintaion of both MLST and SCC*mec* typing data is indispensible for determining the nomeneclature of endemic MRSA clones (Hallin et al. 2007; Enright et al. 2002).

2.6.3 *spa* typing

spa typing is an efficient and highly discriminatory genetic marker that is able to epidemiologically characterize *S. aureus* species and identify closely related ancestral isolates. The *spa* locus of *S. aureus* codes for *S. aureus* protein A (Spa), a 42 KDa anti-phagocytic protein known for its binding capacity to the Fc portion of IgG (Shakeri et al. 2010). The signal sequence (S region) in the N-terminal part of the Spa protein contains five highly homologous IgG-binding units: E, D, A, B and C, each contains approximately 58 amino acids (Baum et al. 2009; Shakeri et al. 2010). On the other hand, the C-terminal part (also known as the X region) consists of two domains: (i) conserved region X_C that binds *S. aureus*' cell wall via the LPXTG-binding motif and (ii) repeat region X_R consists of highly polymorphic internal variable region of 24-27 bp short tandem repeats, which vary not only in number but also with the sequence of the individual repeat units (Baum et al. 2009; Grundmann et al. 2010). Determining the sequence polymorphism in the variable X region constitutes the basis of *spa* typing. Thus, the combination of the different repeats is assigned to a unique *spa* type designated by lower case "t" followed by a number. *spa* types are then clustered into *spa* complexes (*spa*-CCs) using based upon repeat pattern (BURP) algorithm that group clonal relatedness of strains on the basis of repeat duplication/deletion and point mutation events (Strommenger et al. 2006). This single locus sequence-based technique provide portable, unambiguous and biologically meaningful molecular typing data, which have demonstrated their utility for epidemiological purposes such as transmission and outbreak investigations at various geographical levels.

2.6.4 SCC*mec* typing:

Complete characterization of MRSA lineages depends not only on identifying the bacterial genetic background bust also on molecular characterization of the mobile

genetic element (SCC*mec*), responsible for carrying drug resistance determinant *mecA* (Olivera and de Lencastre 2002; Chmelnitsky et al. 2007). For SCC*mec* type determination, several suggested multiplex PCRs have been developed that require lots of optimization and do not always generate concordant data (Ghaznavani-Rad et al. 2010). To date, at least eight major SCC*mec* elements (I-VII) has been identified based on the combination of *mec* and *ccr* sequence complexes (Zhang et al. 2009) with reports identifying up to eleven types (I-XI) (Zong et al. 2011). SCC*mec* elements range in size between 21-67 kb (Chambers and DeLeo 2009) with large SCC*mec* elements types I-III mostly associated with HA-MRSA infections whilst the smaller elements types IV-VI are commonly associated with CA-MRSA infections (Wang et al. 2010; David and Daum 2010; Valsesia et al. 2010; Smyth et al. 2011). CA-MRSA strains have evolved rapidly in the community since they have acquired the small SCC*mec* elements which contain *mecA* gene with or without additional resistant genes and thus are more mobile and disseminate easily among the community. On the other hand, large SCC*mec* types II and III elements contain additional junkyard regions for insertion of genes conferring resistance to non-β-lactam antibiotics and are thus associated with strains having multi-drug resistance (MDR) phenotypes (Zhang et al. 2005; David and Daum 2010). The developed MRSA nomenclature scheme, suggested by the International Union of Microbiology Societies, uses the data of both MLST and SCC*mec* typing, defined as the "clonal type", for reporting circulating MRSA clones (Hallin et al. 2007; Zhang et al. 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Clinical isolates

Between May 2011 and October 2011, a total of 132 non-duplicate *S. aureus* samples were kindly provided by Dr. George Araj from the American University of Beirut Medical Center (AUB-MC). Table 1 demonstrates number of isolates in terms of origin of the specimen. All isolates were confirmed as *S. aureus* by growth on mannitol salt agar (MSA), gram staining, positive catalase reaction, as well as ability to produce coagulase enzyme using SLIDEX® Staph Plus agglutination kit (Biomérieux, France). The samples were streaked on Tryptone Soy Agar (TSA) and stored in Cryobanks at -80°C. The isolates were designated by HST 001-132.

Table1. Demographic distribution of isolates in terms of specimen origin

Specimen Origin	# Found in Isolates (%)
Wound/ cyst/ abscess	53 (40)
Respiratory	38 (29)
Eye	6 (5)
Blood	2 (2)
Catheter	2 (2)
Urine	1 (1)
Others†	30 (21)

† Others: nose, joint fluid, aspirates, throat, drainage, pleural fluid, ear

3.2 DNA Extraction

Isolates were grown overnight on TSA at 37°C. DNA extraction was done using Nucleospin® Tissue (Macherey-Nagel, Germany) according to manufacturer's instructions. The lysates were then stored at -20°C until needed.

3.3 16S rDNA, PVL, *mecA* genes amplification

Polymerase chain reaction (PCR) assays were performed on a PerkinElmer GeneAmp 9700 thermal cycler (PerkinElmer, Wellesly, Massachusetts). Amplification of the 16S rRNA, PVL and *mec*A genes were done using primers described by McClure et al. 2006 (Table 2). The amplification reaction contained 1.5µl of template DNA in a final volume of 25µl containing 0.4, 0.8 and 0.8µM for the primers specific for the 16S rRNA, *lukS-PV*, and *mecA* genes respectively with 2U of AmpliTaq Fermentas, 1.5mmol.l^{-1} $MgCl_2$, 1.6X Taq buffer, 0.2mM of each deoxynucleoside triphosphate (dNTP). The thermocycling conditions were set at 94°C for 5min followed by 10 cycles of 94°C for 45s, 55°C for 45s, and 72°C for 75s and 25 cycles of 94°C for 45s, 50°C for 45s, and 72°C for 75s and finally soaked at 20°C. The expected PCR amplicons were 756, 433, and 310 bp for the 16S rRNA, *lukS-PV*, and *mecA* gene respectively. The fragments were visualized by 7µl of ethidium bromide staining using 1.5% agarose gel using with 1x Tris-Acetic Acid-EDTA (TAE) buffer at 100V.

Table2. Primers used for the amplification of the 16S rRNA, PVL and *mec*A genes.

Primer	Orientation	Oligonucleotide sequence (5'→3')	Product Size (bp)
Staph 756	Forward	AACTCTGTTATTAGGGAAGAACA	756
	Reverse	CCACCTTCCTCCGGTTTGTCACC	
LUK-PV	Forward	ATCATTAGGTAAAATGTCTGGACATGATCCA	433
	Reverse	GCATCAAGTGTATTGGATAGCAAAAGC	
MecA	Forward	GTAGAAATGACTGAACGTCCGATAA	310
	Reverse	CCAATTCCACATTGTTTCGGTCTAA	

3.4 Typing of the *spa* locus

3.4.1 Pre-sequencing PCR

Typing of the *Staphylococcus* protein A (*spa)* locus was carried by first amplifying the *spa* gene as described previously (Harmsen et al. 2003; Strommenger et al. 2008). The amplification employed 20pmol of the *spa*-1-for (5'- TAA AGA CGA TCC TTC GGT GAG C -3') and 20pmol of *spa*-1-rev (5'-CAG CAG TAG TGC CGT TTG CTT -3') primers in a 20µl mixture containing 2µl DNA, 200µM dNTPs, 5µl of 10-fold concentrated PCR Buffer II (Applied Biosystems), $MgCl_2$ 1.5mM, and 1.25U of AmpliTaq DNA polymerase (Applied Biosystems). Thermal cycling reactions consisted of an initial denaturation (12min at 95°C) followed by 30 cycles of denaturation (30s at 94°C), annealing (30s at 60°C), and extension (60s at 72°C), with a single final extension (10min at 72°C). The resulting DNA fragments were visualized by 7µl of ethidium bromide staining on 1% agarose gel using 1X TAE buffer at 100V.

3.4.2 EXOSAP-IT

To purify the pre-sequencing PCR products, 4µl of EXOSAP-IT (usb®) enzyme was added to 10µl of the PCR products. The reactions consisted of 37°C for 30min, 80°C for 15min and stored at 20°C.

3.4.3 Sequencing PCR

The amplicons previously *spa*-typed were sequenced using the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The sequencing reaction was conducted using 4µl of 5X diluted Bigdye® (Applied Biosystems) premix, 3µl of 1.2µM sequencing primer, and 3µl of the cleaned PCR product in a total volume of 10µl. PCR cycling consisted of initial denaturation step at 96˚C for 1min followed by 26 cycles of denaturation (96˚C for 1s), annealing (50˚C for 5s) and extension (60˚C for 4min).

3.4.4 DNA Precipitation and Loading

The sequencing products were purified using DyeEx® 2.0 Spin Kit (Qiagen, Hilden, Germany). The samples were dried in a vacuum centrifuge, and then 10µl of HiDi formamide were added (Applied Biosystems) as loading media for sequencing electrophoresis on an ABI 3500 Genetic Analyzer. The software Ridom StaphType™ v 2.2.1 (Ridom GmbH, Würzburg, Germany) was used for *spa* sequence analysis.

3.5 MLST

For amplification of the seven housekeeping genes, a PCR was performed in a total volume of 20µl using the 7 housekeeping genes primers for amplification as described by Enright et al. 2002 (Table 3). The reaction contained 2µl DNA, 200µM dNTPs, 10pmol of each primer, 5µl of 10-fold concentrated PCR Buffer II (Applied Biosystems), $MgCl_2$ 1.5mM, and 1.25U of AmpliTaq DNA polymerase (Applied Biosystems). Thermal cycling reactions consisted of an initial denaturation (12min at 95°C) followed by 30 cycles of denaturation (30s at 94°C), annealing (30s at 55°C), and extension (60s at 72°C), with a single final extension (10min at 72°C). The bands were visualized by ethidium bromide staining on 1% agarose gel using 1X TAE buffer. MLST types were assigned by submitting the sequences to the *S. aureus* database on the website (http://www.mlst.net/) and eBURST v3 software was used for MLST sequence analysis and clustering.

Table3. Primers used for the amplification of the seven housekeeping genes.

Primer	Orientation	Oligonucleotide sequence (5'→3')
arcC	Forward	ATTCACCAGCGCGTATTGTC
	Reverse	AGGTATCTGCTTCAATCAGCG
aroE	Forward	ATCGGAAATCCTATTTCACATTC
	Reverse	GGTGTTGTATTAATAACGATATC
Glp	Forward	CTAGGAACTGCAATCTTAATCC
	Reverse	TGGTAAAATCGCATGTGCAATTC
gmk	Forward	ATCGTTTTATCAGGACCATCTG
	Reverse	TCATTAACTACAACGTAATCGTA
Pta	Forward	GTTAAAATCGTATTACCTGAAGG
	Reverse	GACCCTTTTGTTGAAAAGCTTAA
Tpi	Forward	TCGTTCATTCTGAACGTCGTGAA
	Reverse	TTTGCACCTTCTAACAATTGTAC
yqiL	Forward	CAGCATACACACCTATTGGC
	Reverse	CGTTGAGGAATCGATACTGGAAC

3.6 PFGE

Isolates subjected to MLST typing were also typed using PFGE technique to reveal a banding pattern on the gel. PFGE was done according to Georing and Winters (1992) with some modifications. In brief, after suspending the pellet with TEN buffer followed by EC buffer, samples were held at 55°C for about 10min. A 2.0ml aliquot suspension was then mixed with 0.5ml low melting agarose (GE Healthcare) and 25µl Lysostaphin (2mg/ml [Sigma]). Inserts were then casted and left to harden for 10-15min and then placed in 4ml EC buffer and 10-15µl RNase and incubated at 37°C for at least 2h. EC buffer is then decanted and replaced by 4mL TE buffer and 20µL Proteinase K and left overnight at 50˚C. A small slice was cut and the remaining of the plug was stored at 4˚C for later usage. The slice was soaked in 500µL 1x buffer A (Roche) and washed for 30min at 4˚C. The process was repeated 4x with prepared 10X buffer A and kept overnight at 4˚C. The buffer was discarded and replaced with a fresh 200µL 1x buffer A (Roche) and 1µL of 5000U *Sma*I enzyme and incubated for 4h at 25˚C in a thermoblock. The slice was then washed twice with 200µL 0.1x buffer A (Roche) for 15min each time to finally stop the reaction with 20µL of stop mix (0.25% bromophenol blue and 0.1% SDS), and stored at 4˚C. The bands were resolved with a 1% agarose gel

in 0.5x Tris-Boric Acid-EDTA (TBE) and run in the CHEF-DRIII System electrophoresis apparatus (Bio-Rad, Hercules, CA) using 0.5x TBE with an initial time 5s and a final time 50s at 14°C for 23.5hrs. The bands were then visualized under UV (SYNGENE-G:box) after staining with EtBr. Cluster analysis was performed with GelCompar II® software (Applied Maths, Sint-Martens-Latem, Belgium) using dice coefficient and visualized as a dendogram by the unweighted pair group method (UPGMA), using average linkages with 1% tolerance and 1% optimization settings. A similarity cutoff of 80% was used to define a cluster.

3.7 SCC*mec* subtyping

The conditions for the multiplex PCR assay were first optimized by using the following reference strains NCTC 10442, N315, 85/2082, JCSC 4744, JCSC 217, JCSC 47882 and WIS as described by Ito et al. (2001) and Zhang et al. (2005). The protocol was adapted from Zhang et al. 2005. The optimal cycling conditions were the following: 95°C for 15min; 30 cycles of 94°C for 30s, 57°C for 1.5min, and 72°C for 1.5min; and a final extension at 72°C for 10min. For multiplex PCR, a Qiagen® multiplex PCR kit was used. Reaction mixtures contained 1µg of chromosomal template; 25µl master mix with 3mM $MgCl_2$, 5µl primer mix (2mM in TE buffer for each primer) and RNase-free water to a final volume of 50µl. The PCR products (7µl) were resolved in a 1.8% (w/v) Metaphor (Lonza, Rockland, ME, USA) agarose gel in 0.5% Tris-borate-EDTA buffer (Bio-Rad, Hercules, CA) at 80V/cm for 1h and were visualized with ethidium bromide. Table 4 summarizes the PCR primer sets selected based on specificity, compatibility and ability to target fragments of SCC*mec* types I–V (Zhang et al. 2005; Ito et al. 2001).

Table4. Primers used for the simultaneous identification of SCC*mec* elements of MRSA by multiplex PCR as described by Zhang et al. 2005

Primer	Orientation	Oligonucleotide sequence (5'→3')	Product Size (bp)
Type I	Forward	GCTTTAAAGAGTGTCGTTACAGG	613
	Reverse	GTTCTCTCATAGTATGACGTCC	
Type II	Forward	CGTTGAAGATGATGAAGCG	398
	Reverse	CGAAATCAATGGTTAATGGACC	
Type III	Forward	CCATATTGTGTACGATGCG	280
	Reverse	CCTTAGTTGTCGTAACAGATCG	
Type IVa	Forward	GCCTTATTCGAAGAAACCG	776
	Reverse	CTACTCTTCTGAAAAGCGTCG	
Type IVb	Forward	TCTGGAATTACTTCAGCTGC	493
	Reverse	AAACAATATTGCTCTCCCTC	
Type IVc	Forward	ACAATATTTGTATTATCGGAGAGC	200
	Reverse	TTGGTATGAGGTATTGCTGG	
Type IVd	Forward	CTCAAAATACGGACCCCAATACA	881
	Reverse	TGCTCCAGTAATTGCTAAAG	
Type V	Forward	GAACATTGTTACTTAAATGAGCG	325
	Reverse	TGAAAGTTGTACCCTTGACACC	
MecA147	Forward	GTGAAGATATACCAAGTGATT	147
	Reverse	ATGCGCTATAGATTGAAAGGAT	
mecI	Forward	CCCTTTTTATACAATCTCGTT	146
	Reverse	ATATCATCTGCAGAATGGG	
IS1272	Forward	TATTTTTGGGTTTCACTCGG	1,305
mecR1	Reverse	CTCCACGTTAATTCCATTAATACC	
ccrAB-β2	Forward	ATTGCCTTGATAATAGCCITCT	
ccrAB-α2	Reverse	AACCTATATCATCAATCAGTACGT	700
ccrAB-α3	Reverse	TAAAGGCATCAATGCACAAACACT	1,000
ccrAB-α4	Reverse	AGCTCAAAAGCAAGCAATAGAAT	1,600
ccrC	Forward	ATGAATTCAAAGAGCATGGC	336
	Reverse	GATTTAGAATTGTCGTGATTGC	

3.8 Antibiotic susceptibility testing

All 132 *S. aureus* isolates were tested against antibiotic resistance by the Kirby-Baüer disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), 2010. Isolates were streaked on Müeller-Hinton agar (Oxoid) and incubated at 37˚C for 24hrs with the following antibiotics: augmentine, cephalothin, ciprofloxacin, clindamycin, erythromycin, teicoplanin, tetracycline, trimethoprim-sulfamethaxole, gentamicin, ampicillin and rifampin. Isolates collected from urine were tested additionally against nitrofurantoin and norfloxacin. Table 5

summarizes the zone of inhibition (ZOI) for each antibiotic along with the concentration (µg) as recommended.

Table5. Zone of inhibition (mm) for the tested antibiotics according to CLSI, 2010

Antibiotic (µg)	ZOI (mm)			Antibiotic (µg)	ZOI (mm)		
	S	I	R		S	I	R
Cephalothin (30)	≥18	15-17	≤14	**Tetracycline (30)**	≥19	15-18	≤14
Ciprofloxacin (5)	≥21	16-20	≤15	**SXT (1.25/3.75)**	≥16	11-15	≤10
Clindamycin (2)	≥21	15-20	≤14	**Rifampin (5)**	≥20	17-19	≤16
Erythromycin (15)	≥23	14-22	≤13	**Cefoxitin (30)**	≥22	__	≤21
Teicoplanin (30)	≥14	11-13	≤10	**Gentamicin (10)**	≥15	13-14	≤12

CHAPTER FOUR

RESULTS

4.1 16S rRNA, PVL, *mecA* genes amplification

All 132 *S. aureus* isolates were confirmed to be Staph through the amplification of the 16S rRNA gene and tested for being MSSA or MRSA and being PVL positive or not through testing *mecA* and *lukS/F-PV* genes respectively. Accordingly, triplex PCR assay may yield 3, 2 or 1 band/s. The first band of 756bp size corresponds to the partial amplification of the 16S rRNA and must be present for all *Staphylococcus* isolates. The second band is a 433bp band that corresponds to the amplification product of the PVL gene. The third band is a 310bp band that corresponds to the amplification product of the *mecA* gene (Figure 1). Table 6 summarizes the results of the triplex PCR for all 132 isolates showing that 29.5% (39 samples) of the isolates were MRSA and 70.5% (93 samples) were MSSA. 100 isolates were PVL negative (75.8%) and the remaining 24.2% (32 isolates) were PVL positive.

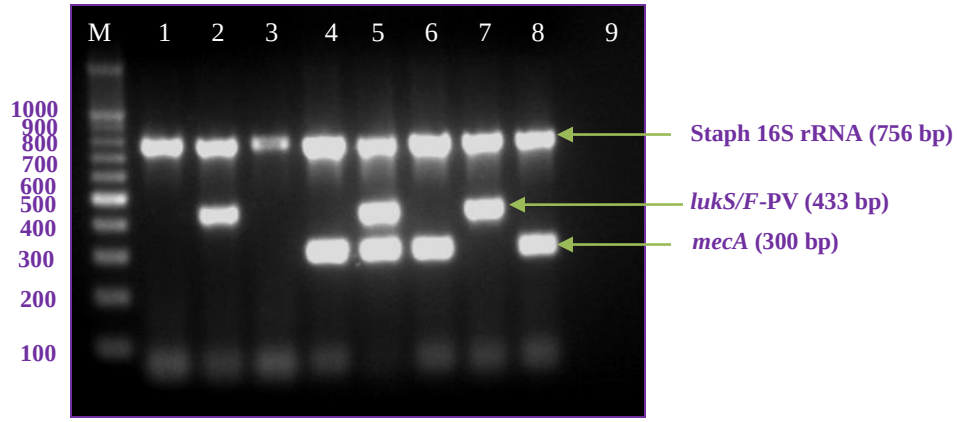

Fig1. Agarose gel electrophoresis showing representative PCR products after 16S rRNA, PVL & *mecA* genes amplification. The first lane shows 100bp DNA marker (Fermentas); lanes 1-8 show PCR products of the three genes of different isolates. Lane 9 is the negative control.

Table6. Distribution of isolates among MRSA and MSSA, PVL positive and PVL negative

Organism		Number of Isolates (%)
MSSA		**93 (70.5)**
	PVL positive	11 (11.8)
	PVL negative	82 (88.2)
MRSA		**39 (29.5)**
	PVL positive	21 (53.8)
	PVL negative	18 (46.2)

4.2 SCC*mec* typing

SCC*mec* subtyping for MRSA isolates showed the predominance of SCC*mec* typeIV (85%) followed by typeV (13%) (Figure2).

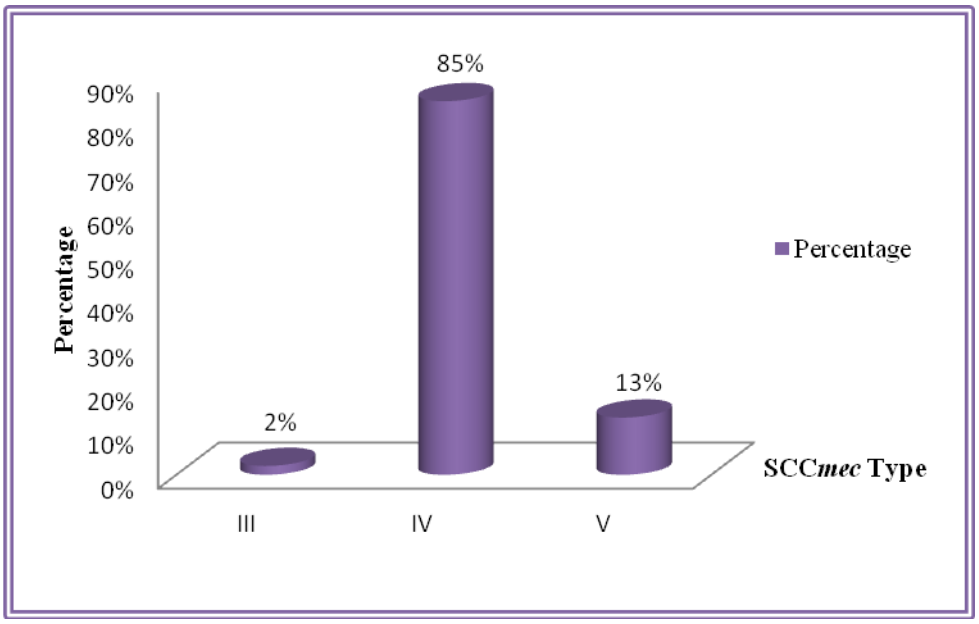

Fig2. Distribution of SCC*mec* types among MRSA isolates. Type IV shows the highest percentage (85%).

4.3 *spa* typing

All 132 *S. aureus* isolates showed bands ranging in size between 250-400bp after *spa* gene amplification (Figure 3). All isolates were typeable and analysis using Ridom Staph Type version 2.2.1 (Ridom GmbH, Würzburg, Germany) revealed 71 different *spa* types in this study. The most common encountered *spa* types were t021, t044 and t267 representing 6%, 5% and 5% of the studied isolates, respectively (Figure 4). 53 different *spa* types were identified in the MSSA isolates compared to 25 in the MRSA isolates. The most common *spa* type within the MSSA was t021 (8%) compared to t044 (13%) within the MRSA isolates (Figures 5 and 6). It is noteworthy that 9 new *spa* types were identified in this study and were designated *spa* types: t9127, t9128, t9129, t9804, t9805, t9806, t9830, t9831 and t9832 with four belonging to *spa* CCs (two representing group founders) while the remaining five being singletons (Table 7). Using BURP analysis, *spa* types were clustered into 40 different groups, with 15 groups having more than one *spa* type and 25 singletons (Figures 7 and 8). Clustering parameters excluded *spa* types with fewer than five repeats and groups *spa* types to the same *spa*-CC if the cost was less than or equal to six. Four strains were excluded (*spa* types t132, t380 and t386) and out of the fifteen *spa* CCs, eight groups had designated group founders (*spa* CC306/002, *spa* CC084, *spa* CC304/121, *spa* CC021, *spa* CC186/786/690, *spa* CC044, *spa* CC032 and *spa* CC267). *spa* CC084 as well as five clusters with no founder had comprised only MSSA strains. MRSA isolates belonging to *spa* CC032 and *spa* CC044 all harbored SCC*mec* type IV, while those belonging to *spa* CC186/786/690 and *spa* CC304/121 had SCC*mec* type IVa and those belonging to *spa* CC267 harbored SCC*mec* type V.

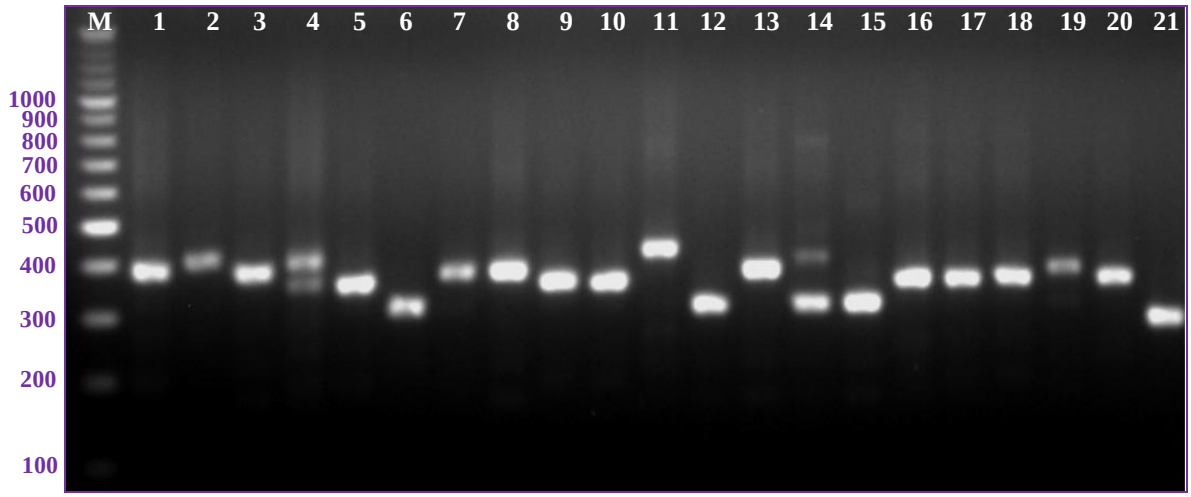

Fig3. Agarose gel electrophoresis showing representative PCR products after *spa* amplification. The first lane shows 100bp DNA marker (Fermentas); lanes 1-21 show PCR products of the amplified *spa* gene with sizes between 250bp and 400 bp

Table7. Ridom repeat succession for the novel 9 *spa* types detected in this study.

Ridom *spa* Type	# of Isolates	*mecA*	PVL	Ridom Repeat Succession	*spa*-CC
t9804	1	MSSA	Neg.	08-16-34-02-43-34-16-16-34-02-16	Singletons
t9805	2	MSSA MSSA	Pos. Neg.	26-22-10-17-20-17-12	Singletons
t9806	1	MSSA	Neg.	7-17-12-23-02-12-23	No founder
t9127	1	MSSA	Neg.	07-23-12-34-34-12-36-23-02-12-23	84
t9128	1	MSSA	Neg.	26-23-34-23-31-05-17-17-25-16-28	Singletons
t9129	1	MSSA	Neg.	7-16-21-17-34-34-34-33-34	267
t9830	1	MSSA	Neg.	04-17-34-17-32-23-24-24	Singletons
t9831	2	MRSA	Neg.	07-23-12-12-20-17-12-12-12	No founder
t9832	2	MRSA	Neg.	125-13-23-31-29-25-17-25-16-28	Singletons

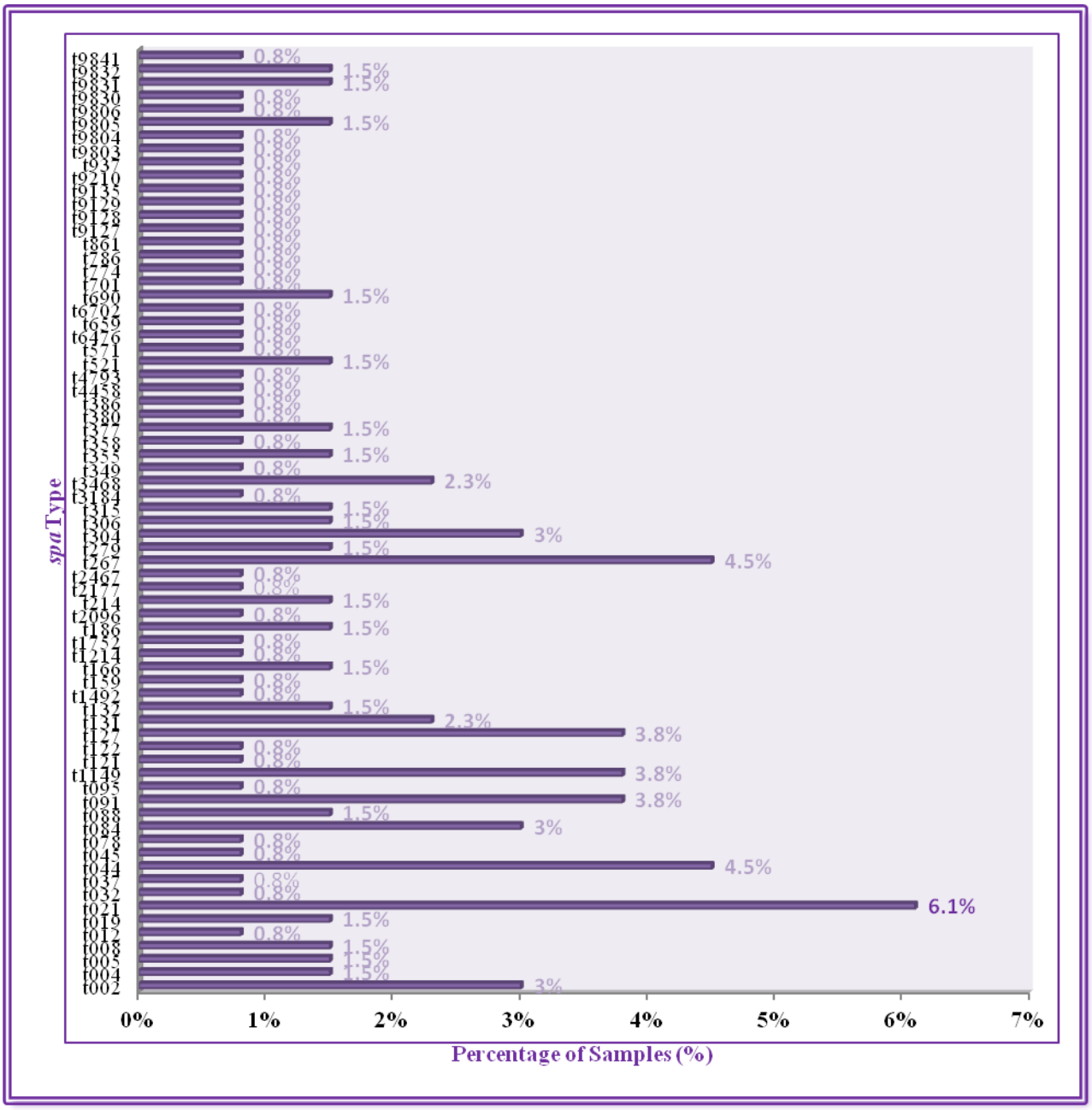

Fig4. Percentage of the 71 *spa* types representing all 132 isolates undertaken in this study with t021 being the predominant *spa* type (6.1%) followed by t267 and t044 (4.5% each).

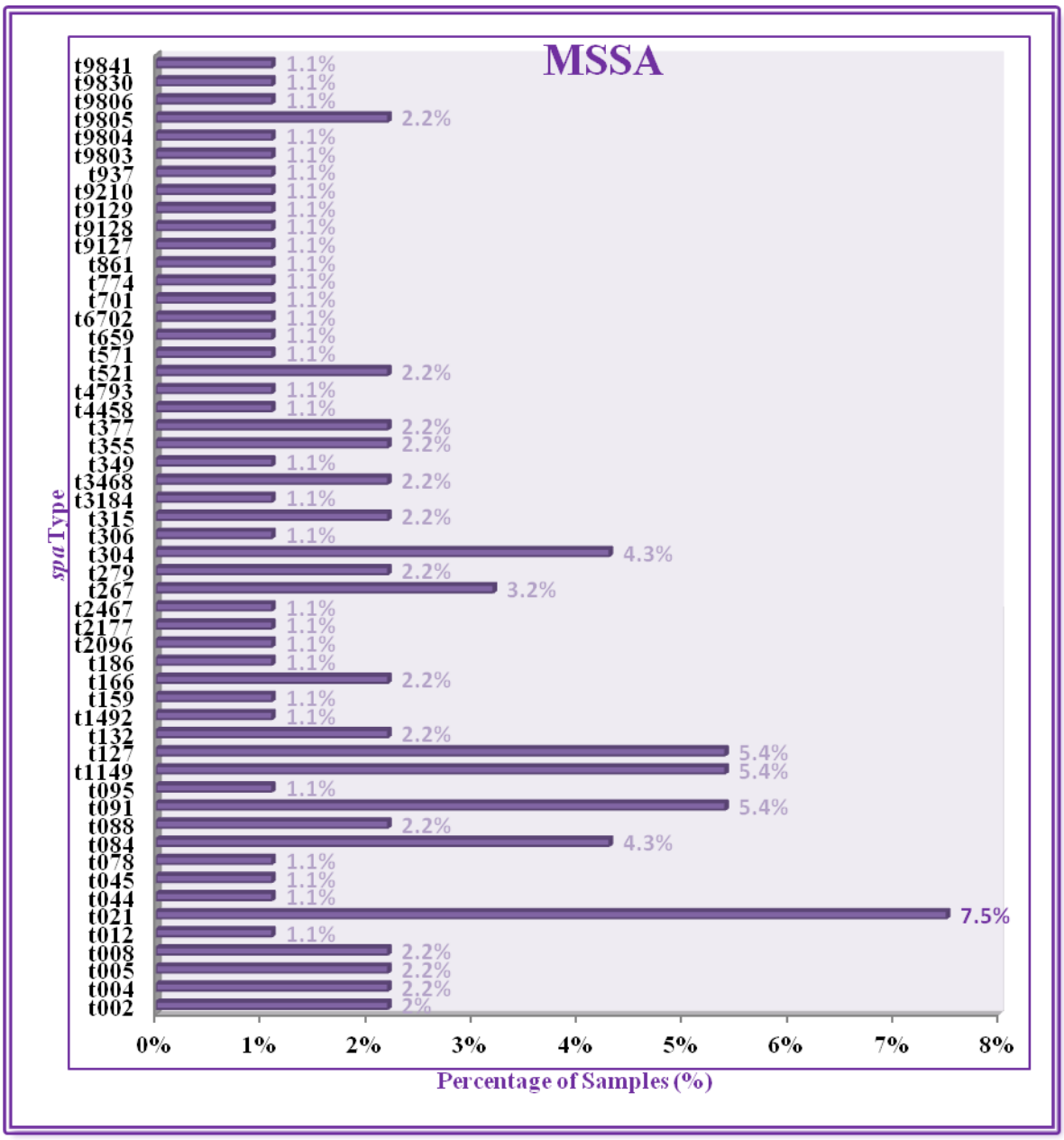

Fig5. Percentage distribution of the different *spa* types in the MSSA lineage (n=93) with t021 being the predominant *spa* type (7.5%).

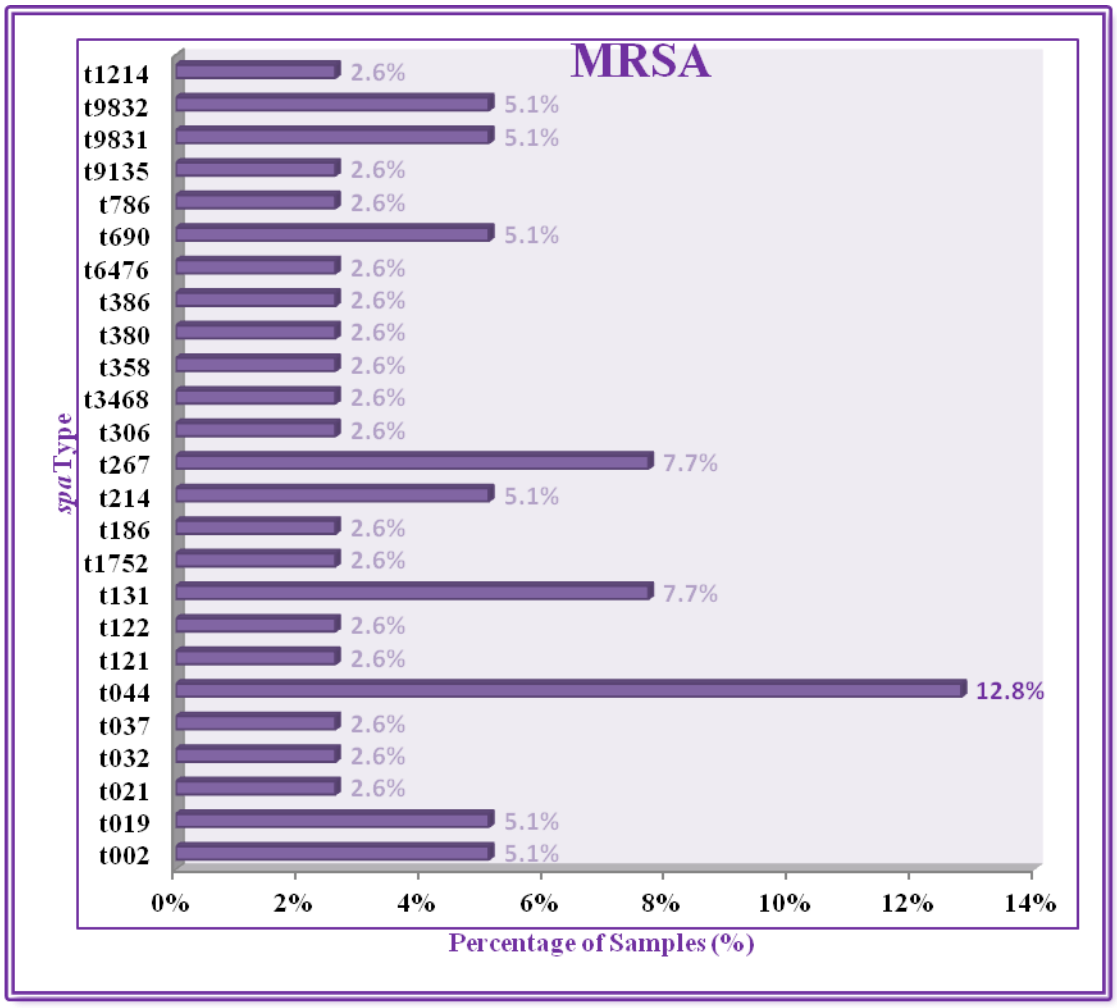

Fig6. Percentage distribution of the different *spa* types in the MRSA lineage (n=39) with t044being the predominant *spa* type (12.8%).

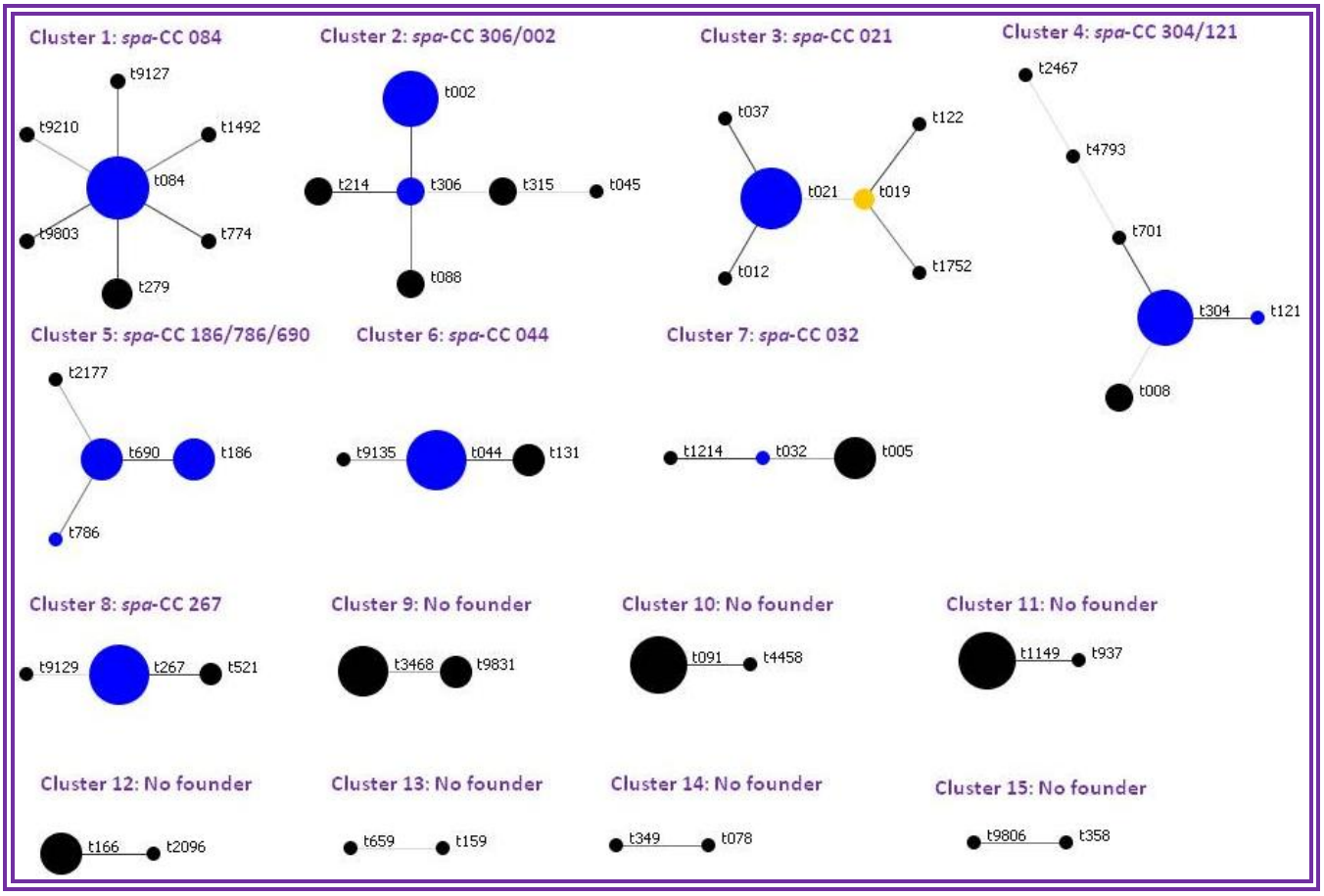

Fig7. Population snapshot based on BURP analysis of 132 isolates. BURP grouping using default parameters resulted in 15 *spa* CCs with 8 having group founders, 25 singletons, and 4 excluded *spa* types. Each dot represents a unique *spa* type. The diameter of the a dot is proportional to the quantity of the corresponding *spa* type. Blue dots represent primary group founders, defined as the *spa* type(s) with the highest founder score within a CC, while yellow dots represents major subgroup founders.

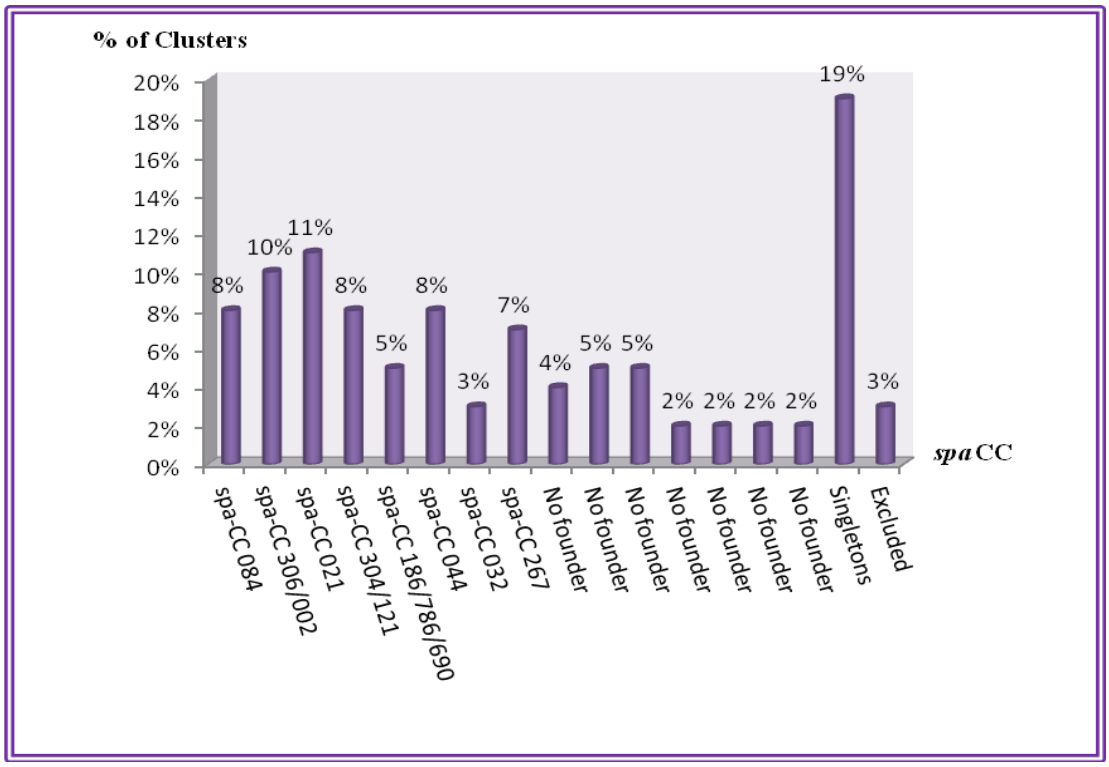

Fig8. Clustering *spa* types by BURP showing the percentage of samples belonging to each *spa*-CC.

4.4 MLST

Thirty six isolates representing all *spa* CCs were MLST-typed. 25 allelic profiles were generated in this study representing STs. There were seven major MRSA clones defined as isolates with the same ST and SCC*mec* type. These clones were associated with complexes CC5, CC22, CC30, CC72, CC80 and CC239. On the other hand, twenty MSSA clones were identified and those were associated with complexes CC1, CC6, CC7, CC8, CC15, CC25, CC30, CC45, CC88, CC97, CC121, CC361, CC398, CC641, CC770 and CC789. The predominant clones in this study were ST80-MRSA-IV (7.7%) followed by ST30-MSSA (6.1%) (Table 8). In order to view actual evolutionary events among the observed STs, sequences of the seven housekeeping genes were concatenated into a single sequence followed by alignment against all other sequences (Figure 9). 100% confidence levels were observed in STs that belong to the same MLST-CC (e.g. ST15 and ST199 belonging to CC15, and ST30 and ST34 belonging to CC30). 99% and 95% confidence levels were observed in STs that share single locus variants and belong to different MLST-

CCs (e.g. ST5 and ST641, ST789 and ST7, and ST239 and ST8). Low confidence levels (39%) were observed in STs that belong to different MLST-CCs (e.g. ST361 and ST80).

Table8. Overview of the major MRSA and MSSA *spa* types and their corresponding MLST clones.

Clone	*spa* type (%)	*spa*-CC	PVL	MLST	MLST-CC
ST5-MRSA-IV	t002 (1.5%)	306/002	Neg.	5	5
	t214 (1.5%)	306/002	Neg.	5	5
ST6-MSSA	t304 (3%)	304/121	Neg.	6	6
ST361-MSSA	t315 (1.5%)	306/002	Neg.	361	361
ST199-MSSA	t084 (3%)	84	Neg.	199	15
	t9210 (0.8%)	84	Neg.	199	15
ST15-MSSA	t774 (0.8%)	84	Neg.	15	15
ST8-MSSA	t008 (1.5%)	304/121	Pos.	8	8
ST30-MSSA	t012 (0.8%)	21	Neg.	30	30
	t021 (5.3%)	21	Pos.	30	30
ST30-MRSA-IV	t019 (1.5%)	21	Pos.	30	30
ST34-MSSA	t2096 (0.8%)	No founder	Neg.	34	30
	t166 (1.5%)	No founder	Neg.	34	30
ST80-MRSA-IV	t021 (0.8%)	21	Neg.	80	80
	t044 (3.8%)	44	Pos.	80	80
	t131 (2.3%)	44	Pos.	80	80
	t6476 (0.8%)	Singletons	Pos.	80	80
ST720-MSSA	t659 (0.8%)	No founder	Neg.	720	121
ST1-MSSA	t127 (3.8%)	Singletons	Neg.	1	1
ST22-MRSA-IV	t9832 (1.5%)	Singletons	Neg.	22	22
ST508-MSSA	t861 (0.8%)	Singletons	Neg.	508	45
ST46-MSSA	t132 (1.5%)	Excluded	Neg.	46	45
ST88-MSSA	t186 (1.5%)	186/786/690	Neg.	88	88
ST72-MRSA-IV	t3468 (0.8%)	No founder	Neg.	72	72
ST72-MRSA-V	t9831 (1.5%)	No founder	Neg.	72	72
ST97-MSSA	t9129 (0.8%)	267	Neg.	97	97
	t044 (0.8%)	44	Neg.	97	97
ST291-MSSA	t937 (0.8%)	No founder	Neg.	291	398
	t1149 (3.8%)	No founder	Neg.	291	398
ST7-MSSA	t091 (3.8%)	No founder	Neg.	7	7
ST789-MSSA	t9806 (0.8%)	No founder	Neg.	789	789
ST770-MSSA	t377 (1.5%)	Singletons	Neg.	770	770
ST641-MSSA	t005 (1.5%)	32	Neg.	641	641
ST25-MSSA	t078 (0.8%)	No founder	Neg.	25	25
ST239-MRSA-III	t037 (0.8%)	21	Neg.	239	239
ST1995-MSSA	t349 (0.8%)	No founder	Neg.	1995	25

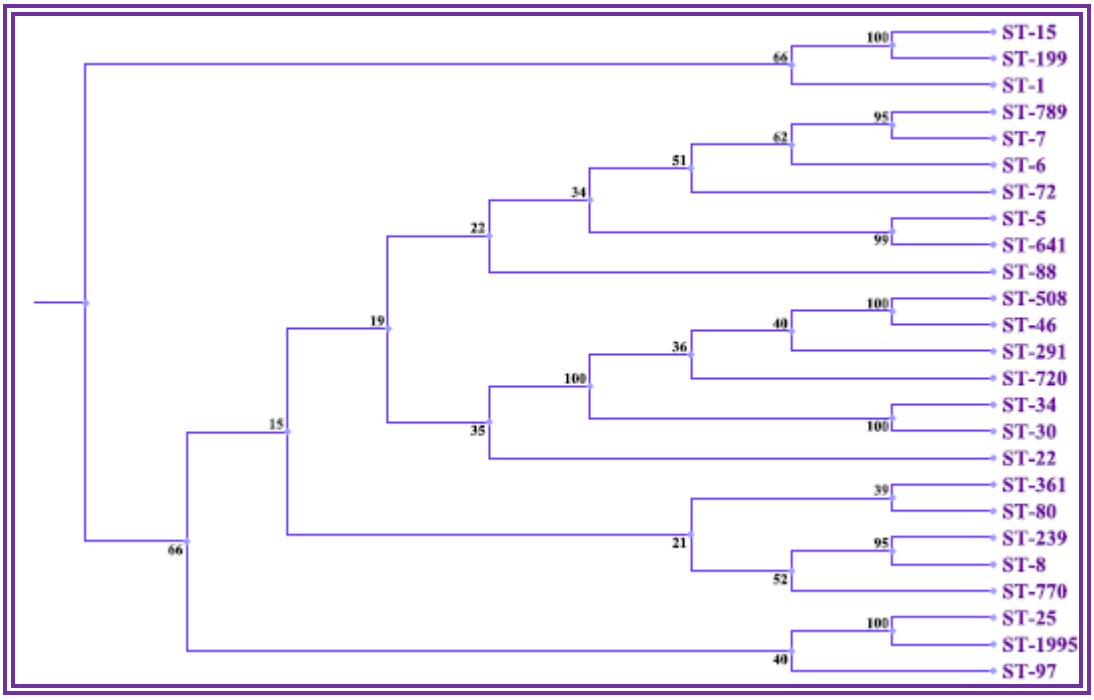

Fig9. Neighbor-Joining tree of the representative STs. Sequences for the variable sites from the seven housekeeping gene fragments were concatenated into a single sequence.

4.5 PFGE

Selected MLST-typed samples were subjected also to PFGE typing. All 36 isolates were typeable by *Sma*I macrorestriction and produced different macrorestriction patterns according to the criteria defined by Tenover et al. (1995). Thirty two groups were defined by employing 80% similarity as cutoff with only two groups containing more than one isolate. Clusters were designated by numbers from 1 to 32 (Figure10).

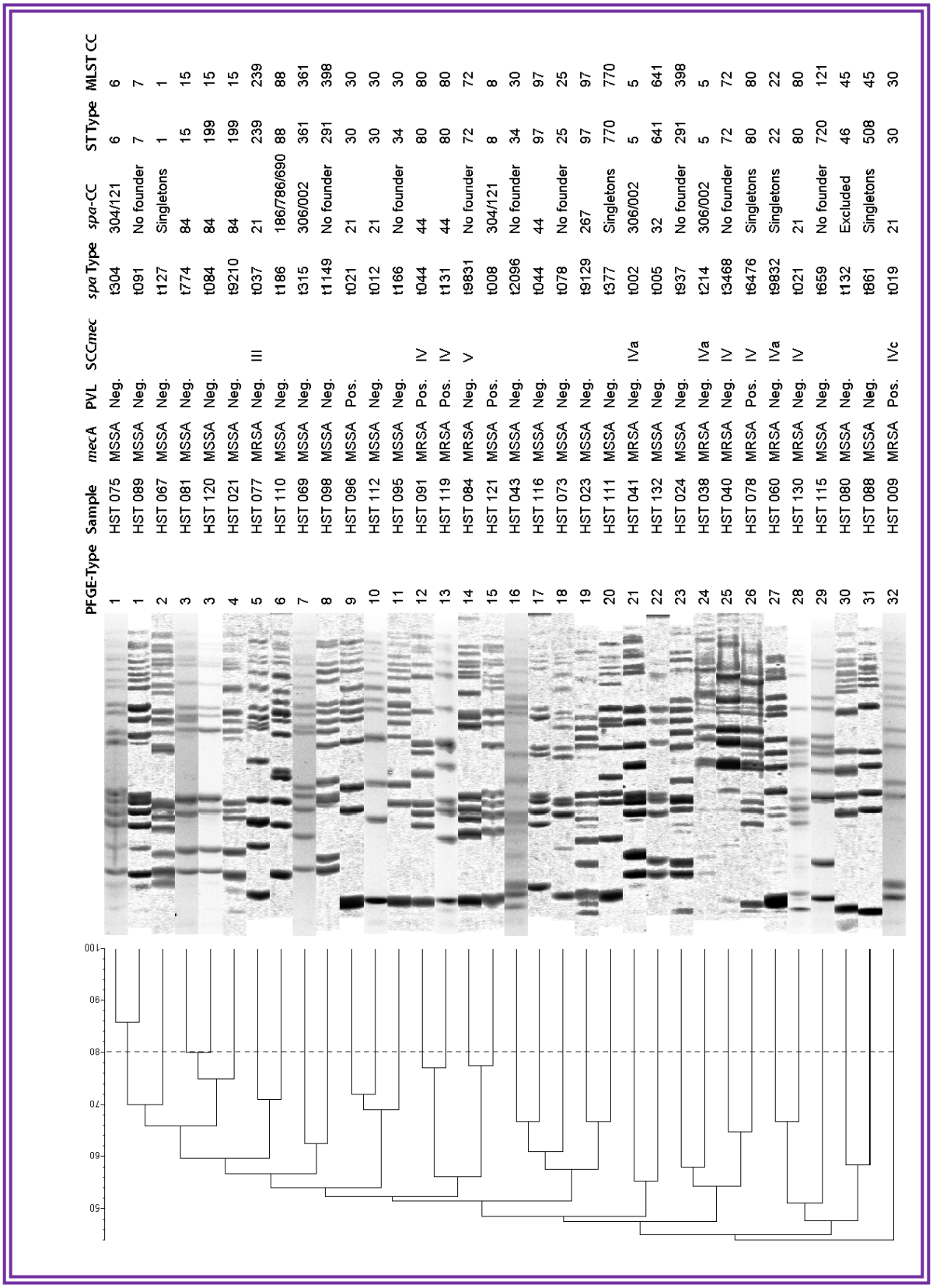

Fig10. Dendrogram of PFGE clusters and genotypic relationships of *S. aureus* isolates. *Sma*I macrorestriction patterns were analyzed using the Dice coefficient and visualized by unweighted-pair group method, using average linkages with 1% tolerance and 1% optimization settings. The similarity cutoff of 80% is indicated by a vertical line. PFGE groups determined by cluster analysis are numbered from 1-32. *spa* types, *spa*-CC, ST type and MLST-CC are also included.

4.6 Antibiotic susceptibility testing

Seventy five (57%) out of the 132 isolates were sensitive to all tested antibiotics. Among the remaining 57 isolates (43%), resistance was detected to one or more of the antibiotics. Results obtained showed that 14% of isolates were resistant to augmentin, cephalothin and oxacillin and were also shown to be methicillin-resistant. On the other hand, the multidrug resistant profiles within the MSSA included resistance against clindamycin and erythromycin (5%) in an inducible manner. It is noteworthy that all isolates were sensitive to teicoplanin, vancomycin and rifampin. Table 9 below summarizes the percentage of the 19 generated resistant profiles in this study as well as their distribution among MRSA and MSSA.

Table9. Percentage distribution of resistant profiles observed among MRSA and MSSA.

Resistant Profile (%)	# among MSSA	# among MRSA
ERY (0.7)	1	0
TET (4.5)	5	1
CIP (1.5)	2	0
CLI, ERY (4.5)	6	0
CIP, ERY (0.7)	1	0
CIP, TET (0.7)	1	0
CIP, CLI (0.7)	1	0
TET, SXT (0.7)	1	0
CLI, ERY, TET (0.7)	1	0
KF, OXA, TET (0.7)	0	1
AUG, KF, OXA (14.4)	0	19
AUG, KF, OXA, TET (4.5)	0	6
AUG, KF, ERY, OXA (0.7)	0	1
AUG, KF, CIP, ERY, OXA (2.3)	0	1
AUG, KF, CIP, CLI, ERY, OXA (2.3)	0	3
AUG, KF, CLI, ERY, OXA, TET (1.5)	0	2
AUG, KF, CIP, CLI, ERY, OXA, TET, SXT (0.7)	0	1
AMP, AUG, KF, OXA (0.7)	0	1
AMP, AUG, KF, OXA, TET (0.7)	0	1

CHAPTER FIVE

DISCUSSION

In this study, 132 clinical *S. aureus* isolates were collected between May-October 2011 and genotyped using different molecular techniques including SCC*mec* typing, *spa* typing, MLST and PFGE. Forty percent of the isolates were recovered from wound, cysts and abscesses which are congruent with high prevalence of SSTI infections caused by *S. aureus* (David and Daum 2010). MRSA occurrence among *S. aureus* varies according to the geographical region, with a low frequency (~1%) in some countries in Europe (e.g. Netherlands, Denmark and Sweden) and a high frequency (>60%) in countries such as that observed in US and Japan (Stam-Bolink et al. 2007; Fang et al. 2008; Larsen et al. 2008; Klevens et al. 2007; Tiemersma et al. 2004). The prevalence of MRSA in our study was moderate (29.5%) compared to previous studies done in Lebanon (January 2006-May 2007) showing predominance of MRSA isolates (71.5%) (Tokajian et al. 2010). Although the role of PVL in *S. aureus* pathogenicity remains controversial with a number of studies showing its association with primary skin infections and necrotizing pneumonia, while others reducing its importance as a virulent factor (Brown et al. 2007; Gillet et al. 2002; Lina et al. 1999; Labandeira-Rey et al. 2007; Voyich et al. 2006; Bubeck Wardenburg et al. 2008). In our study, PVL genes were detected in 54% of MRSA and 12% of MSSA. Several conducted studies have shown the prevalence of PVL genes among MRSA compared to MSSA isolates from infections and colonization (Baggett et al. 2004; Martinez-Aguilar et al. 2004) with an increase in the severity of infections in PVL positive MSSA strains being detected (Gillet et al. 2002; Roberts et al. 2008; Salliot et al. 2006; Sola et al. 2007).

SCC*mec* typing, which is important for defining MRSA clones, showed the prevalence of the mobile genetic element SCC*mec* typeIV (85%), which is commonly known to be associated with CA-MRSA infections (Wang et al. 2010; Smyth et al. 2011;

Valsesia et al. 2010). Similar results were also obtained in the study conducted by Tokajian et al. (2010) in Lebanon, where SCC*mec*IV was detected in 88% of MRSA isolates. The predominance of SCC*mec* typeIV in the community is probably due to its small size compared to other SCC*mec* elements which facilitates its transfer to MSSA strains (Robinson and Enright 2004; David and Daum 2010). Several studies have described that PVL along with SCC*mec* typesIV or V are markers for CA-MRSA infections in Europe and USA (Tristan et al. 2007; Vandenesch et al. 2003; Shukla et al. 2004), while in Australia the majority of CA-MRSA were found to be PVL negative (O'Brien et al. 2004). It is worth mentioning that all MRSA isolates, in our study, harboring SCC*mec*IV were PVL positive which agrees with previous results (Tokajian et al. 2010; Khalil et al. 2012; Diep et al. 2004; Deresinski 2005). On the other hand, all (n=5) MRSA isolates harboring SCC*mec*V were PVL negative, and this was in harmony with several other studies conducted in Lebanon and elsewhere (Tokajian et al. 2010; Khalil et al. 2012; Feng et al. 2008; Adler et al. 2012; Chmelnitsky et al. 2007). One isolate in our study harbored SCC*mec*III, which is known to be associated with HA-MRSA (Ma et al. 2002; Yamamoto et al. 2010; Wang et al. 2010), and this isolate was additionally multidrug resistant. Resistance against at least three drugs is associated with the presence of the junkyard region in SCC*mec* element typeIII within which are the genes that confer resistance to non-β-lactam antibiotics (David and Daum 2010).

Using default parameters of BURP, resultant *spa* types were grouped into 15 *spa* CCs and 25 singletons. Seventy one different *spa* types were detected in this study with 53 different *spa* types belonging to the MSSA lineage compared to 25 in the MRSA lineage. This clearly shows the wide diversity of MSSA compared to MRSA that was also shown in the study conducted by Khalil et al. (2012) and Grundmann et al. (2010). The most prevalent *spa* type among MRSA was t044 (12.8%), which is also a common finding from isolates detected in the region (Jordan (10.7%) and Lebanon (37.7%) (Khalil et al. 2012; Tokajian et al. 2010) as well as in Europe (Grundmann et al. 2010; Deurenberg and Stobberingh 2008). On the other hand, the most prevalent *spa* type observed among the MSSA lineage was t021 (7.5%), which was not comparable with the findings of Khalil et al. (2012) and Tokajian et al. (2010), however, this isolate is

commonly found in Ireland (7.1%), Romania (12%), Portugal (4.2%) and other areas in Europe (Grundmann et al. 2010; Deurenberg and Stobberingh 2008).

Thirty six isolates, within which 11 were MRSA and 25 were MSSA, representing all *spa*-CCs were MLST-typed. Twenty five allelic profiles were generated with the most prevalent STs being ST80 (11%) and ST30 (8%), which was in harmony with previous findings in Lebanon (Tokajian et al. 2010). These STs were also prevalent in Jordan (Khalil et al. 2012), Kuwait (Udo et al. 2008; Udo et al. 2010), and Israel (Adler et al. 2010). Seven MRSA clones along with 20 MSSA clones were identified. Two out of the 5 major international PVL positive CA-MRSA clones were detected in our study (European/Middle Eastern CC80:MRSA:IV and Southwest Pacific CC30:MRSA:IV clones). Additionally, 3 out of the 12 known HA-MRSA clones were also detected in the study (Brazilian/Hungarian CC8:MRSA:III, Pediatric CC5:MRSA:IV and UK EMRSA-15 CC22:MRSA:IV clones) (Deurenberg and Stobberingh 2008). Majority of MSSA strains detected represent international pandemic clones belonging to CC1, CC5, CC8, CC121 and CC30 (Strommenger et al. 2006). Enright et al. (2002) has illustrated that the major MRSA lineages (CC5, CC22 and CC30) have emerged from epidemic MSSA lineages.

PFGE analysis of 36 isolates revealed 32 different genotypes when 80% cutoff similarity was employed. This clearly showed the high genetic diversity of the collected *S. aureus* isolates. Several conducted studies have shown the higher diversity detected using PFGE compared to that defined by *spa* typing and/or MLST (Bosch et al. 2010; Cookoson et al. 2007). The need to introduce clustering analysis when conducting PFGE is of high importance to create databases that can be used for long-term epidemiological studies.

Typing results using MLST and PFGE showed that several isolates belonging to the same MLST CCs were assigned to different PFGE clusters; CC30 and CC80 strains were present in more than one PFGE cluster. However, strains that belong to the same PFGE cluster generally belonged to the same MLST CC (e.g. CC15). This suggested that PFGE may have better discriminatory power than MLST for typing of *S. aureus*, which agreed with the findings of Xie et al. (2011) and Blanc et al. (2007).

Clustering SCC*mec* with MLST showed that the PVL positive ST80-MRSA-IV (6.9%) followed by PVL positive ST30-MSSA (5.3%) were the predominant types. The majority of the MRSA in the PVL positive ST80-MRSA-IV clone harbored *spa* type t044 (3.8%) followed by t131 (2.3%) and t6476 (0.8%). The study done by Tokajian et al. (2010) has revealed that the majority of isolates (37.7%) belonged to ST80-MRSA-IVc and were *spa* t044. This was also in harmony with other conducted studies showing of ST80 clone in the region (Khalil et al. 2012; Udo et al. 2008; Udo et al. 2010; Adler et al. 2010). This clone, known as the European clone (Goering et al. 2009; Deurenberg and Stobberingh 2008), has most probably disseminated from the Middle East. Goering et al. (2009) showed that the majority of 294 collected CC80:ST80:IV cases has family relationships in the Middle East. Since Denmark is a country with very low prevalence of MRSA, it is most likely that this clone was introduced into the Denmark from immigrants coming from the Middle East (Goering et al., 2009). This was also supported by the results obtained by Maier et al. (2007) who showed that travelers returning from Dead Sea area towards Germany as well as patients having family relationships from Turkey carried this clone. Finally, Francois et al. (2008) has shown that travelers returning from Tunisia and Libya towards Switzerland carried this clone too. Hence, studies that aim at defining the disease burden from this clone in Lebanon and the neighboring countries should be conducted to limit its future spread in the region as well as other areas (Tokajian et al. 2012). Thus, intervention and prevention control measures that aim at systematic decolonization of MRSA carriers, antibiotic stewardship for containing resistance development and improvement of effective vaccine therapies is highly recommended for limiting the overall burden of predominant clones (Skov et al. 2012).

It is noteworthy, that one isolate in our study collected from a three year old female was MSSA and PVL negative and typed as *spa* t044. This isolate is unique since it is usually known to be MRSA (Grundmann et al. 2010; Deurenberg and Stobberingh 2008; David and Daum 2010). The isolate had also distinct allelic profile from the known ST80 (1-3-1-14-11-51-10), where its allelic profile was similar to ST97 (3-1-1-1-1-5-3) with a pulsotype that was different from its MRSA counterpart.

PVL positive ST30-MRSA-IV was detected in our study in 1.5% of isolates which indicated that ST30-MSSA strains acquired the small mobile SCC*mec* typeIV element and disseminated consequently to become community-associated.PVL positive ST30-MSSA clone, commonly known as phage type 80/81, is one of the most remarkable bacterial clones that have caused epidemics in the 20th century. This clone has emerged in the 1950s in the community as well as in hospital settings and was successfully eradicated after the introduction of methicillin into clinical practice (Deurenberg and Stobberingh 2008). However, this clone has reemerged and acquired SCC*mec* typeIV element and became the community acquired ST30-MRSA-IV clone. ST30-MSSA also acquired SCC*mec*II element into a single locus variant of ST30 and was introduced into hospital settings to become ST36-MRSA-II clone (UK EMRSA-16) commonly found in the United Kingdom (Enright et al. 2002; Deurenberg and Stobberingh 2008).

In conclusion, this study provides a comprehensive overview of the molecular epidemiology and genetic diversity of *S. aureus* isolates collected from the largest university hospital in Lebanon. Application of multiple genotypic typing methods provided key information about the clonal relatedness of the isolates that increased our understanding of *S. aureus* clones circulating in Lebanon. The persistent spread of the PVL positive ST80-MRSA-IV clone in Lebanon as well as neighboring countries calls for novel approaches for infection control measures that would limit its spread. The wide diversity observed within MRSA and MSSA clones raises the question whether these clones will eventually replace the prevalent local clones or not. Hence, continuous surveillance studies with the aid of molecular typing techniques, continues to be of great importance for understanding the local epidemiology of *S. aureus*. Limitations of our study lied in the small number of isolates as well as lack of information about the clinical history of patients that would have given us insights to track clones disseminating between the hospitals and in the community.

CHAPTER SIX

CONCLUSION

- This study reflects the epidemiological characterization of *S. aureus* isolates collected from the biggest university hospital in Lebanon through employment of four genotypic techniques: SCC*mec*, *spa*, MLST and PFGE.
- High genetic diversity was demonstrated by all of the typing methods.
- SCC*mec* typing of MRSA showed the prevalence (49%) of SCC*mec* typeIV (d, e, f, g and h) followed by IVa (31%), V (13%), IVc (5%) and III (3%).
- *spa* typing revealed 71 different *spa* types with the most common being t021, t044 and t267.
- 53 different *spa* types were identified among MSSA isolates compared to 25 among MRSA isolates.
- Using BURP analysis, *spa* types were clustered into 40 different groups with 15 groups having more than one *spa* type and 25 singletons.
- 9 novel *spa* types were identified: t9127, t9128, t9129, t9804, t9805, t9806, t9830, t9831 and t9832.
- 25 distinct allelic profiles were generated by MLST with ST80 (11%) and ST30 (8%) being the predominant STs.
- PFGE aimed at defining 32 PFGE clusters among 36 tested isolates by employing 80% cutoff similarity.
- PFGE has been shown to have better discriminatory power than MLST in typing of *S. aureus*.
- Seven MRSA clones along with twenty MSSA clones were identified.
- PVL positive ST80-MRSA-IV and ST30-MSSA clones were the major disseminating MRSA and MSSA clones respectively.

- Nineteen resistant profiles were obtained in this study. MRSA isolates were mainly resistant to augmentin, cephalothin and oxacillin, while MSSA to clindamycin and erythromycin.
- Prevalence of SCC*mec* IV (85%) among MRSA isolates suggests that *S. aureus* infection among the Lebanese population is due to CA-MRSA.
- Access to specific and precise typing methods that offer cost effective intervention and prevention strategies is of high importance for continuous surveillance and outbreak investigations of pandemic clones.
- Persistence of ST80-MRSA-IV clone calls for further infection control measures that aids in limiting its regional as well as global spread.
- Employment of larger scale studies that includes large number of local samples as well as patient clinical history is indispensable for understanding the genetic relationships as well as evolution of circulating *S. aureus* in Lebanon.

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ANNEX

List of all the *spa* types found among our isolates, along with their respective *spa* clonal complexes (*spa*-CCs), SCC*mec* subtypes, ST types, MLST CCs and antibiotic resistance pattern.

Sample #	Specimen	PVL	Methicillin	SCC*mec* Subtype	*spa* type	*spa*-CC	ST Type	MLST CC	Antibiotic Resistance
HST 001	Sputum	Neg.	Susceptible		t008	304/121			
HST 002	Blood	Neg.	Susceptible		t9128	Singletons			
HST 003	Pus	Neg.	Susceptible		t9127	084			
HST 004	Wound	Neg.	Susceptible		t084	Singletons			
HST 005	Nose	Neg.	Susceptible		t521	267			
HST 006	Joint Fluid	Neg.	Susceptible		t4458	No founder			
HST 007	Wound	Pos.	Resistant	IV	t9135	044			AUG, KF, OXA
HST 008	Pus	Neg.	Susceptible		t571	Singletons			CLI, ERY
HST 009	Wound	Pos.	Resistant	IVc	t019	021	30	30	AUG, KF, OXA
HST 010	Others	Neg.	Susceptible		t084	084			
HST 011	Sputum	Neg.	Resistant	IV	t032	032			AUG, KF, CIP, CLI, ERY, OXA
HST 012	Pus	Neg.	Susceptible		t279	084			
HST 013	Others	Neg.	Susceptible		t521	267			
HST 014	Others	Neg.	Susceptible		t279	084			
HST 015	Toe	Neg.	Susceptible		t304	304/121			
HST 016	Wound	Neg.	Susceptible		t095	Singletons			
HST 017	Eye	Pos.	Resistant	IV	t131	044			AUG, KF, OXA, TET
HST 018	Eye	Pos.	Resistant	IV	t131	044			AUG, KF, OXA, TET
HST 019	Pus	Neg.	Susceptible		t701	304/121			
HST 020	Tracheal Aspirate	Neg.	Susceptible		t2177	186/786/690			ERY
HST 021	Tracheal Aspirate	Neg.	Susceptible		t9210	084	199	15	
HST 022	Sputum	Neg.	Susceptible		t088	306/002			
HST 023	Sputum	Neg.	Susceptible		t9129	267	97	97	CLI, ERY, TET
HST 024	Others	Neg.	Susceptible		t937	No founder	291	398	
HST 025	Pus	Neg.	Susceptible		t304	304/121			
HST 026	Eye	Neg.	Susceptible		t304	304/121			
HST 027	Sputum	Neg.	Susceptible		t002	306/002			

HST 028	Abdominal	Pos.	Resistant	IV	t002	306/002			AUG, KF, CLI, ERY, OXA, TET
HST 029	Urine	Neg.	Susceptible		t045	306/002			CLI, ERY
HST 030	Wound	Neg.	Resistant	IVa	t786	186/786/690			AUG, KF, OXA
HST 031	Pus	Neg.	Susceptible		t091	No founder			TET
HST 032	Others	Neg.	Resistant	IVa	t214	306/002			AUG, KF, OXA
HST 033	Eye	Neg.	Resistant	IV	t1214	032			AUG, KF, CIP, CLI, ERY, OXA
HST 034	Pus	Pos.	Resistant	IVa	t121	304/121			AUG, KF, CIP, ERY, OXA
HST 035	Pus	Neg.	Resistant	IVa	t186	186/786/690			AUG, KF, ERY, OXA
HST 036	Sputum	Neg.	Susceptible		t088	306/002			
HST 037	Others	Neg.	Susceptible		t1149	No founder			
HST 038	Others	Neg.	Resistant	IVa	t214	306/002	5	5	AUG, KF, OXA
HST 039	Tracheal Aspirate	Neg.	Resistant	IVa	t386	Excluded			AUG, KF, CLI, ERY, OXA, TET
HST 040	Nose	Neg.	Resistant	IV	t3468	No founder	72	72	
HST 041	Tracheal Aspirate	Neg.	Resistant	IVa	t002	306/002	5	5	AUG, KF, OXA
HST 042	Eye	Neg.	Susceptible		t091	No founder			
HST 043	Tracheal Aspirate	Neg.	Susceptible		t2096	No founder	34	30	
HST 044	Tracheal Aspirate	Pos.	Resistant	IVc	t1752	021			AUG, KF, OXA
HST 045	Others	Neg.	Susceptible		t1149	No founder			
HST 046	Wound	Neg.	Susceptible		t1149	No founder			
HST 047	Others	Pos.	Resistant	IV	t380	Excluded			AUG, KF, OXA, TET
HST 048	Pus	Neg.	Susceptible		t1492	084			TET
HST 049	Pus	Pos.	Resistant	IV	t358	No founder			
HST 050	Tracheal Aspirate	Neg.	Susceptible		t132	Excluded			
HST 051	Sputum	Neg.	Susceptible		t9806	No founder	789	789	CIP, TET
HST 052	Wound	Pos.	Resistant	IV	t122	021			AUG, KF, OXA
HST 053	Wound	Pos.	Susceptible		t9803	084			
HST 054	Throat	Neg.	Susceptible		t091	No founder			
HST 055	Nose	Neg.	Susceptible		t9804	Singletons			
HST 056	Others	Neg.	Susceptible		t267	267			
HST 057	Others	Pos.	Resistant	IVa	t306	306/002			AUG, KF, OXA
HST 058	Wound	Neg.	Susceptible		t021	021			

HST 059	Pus	Pos.	Susceptible		t9805	Singletons			
HST 060	Sputum	Neg.	Resistant	IVa	t9832	Singletons	22	22	AMP, AUG, KF, OXA
HST 061	Tracheal Aspirate	Neg.	Resistant	V	t267	267			AMP, AUG, KF, OXA, TET
HST 062	Pus	Neg.	Susceptible		t021	021			
HST 063	Catheter	Neg.	Susceptible		t021	021			
HST 064	Pus	Neg.	Susceptible		t005	032			
HST 065	Pus	Pos.	Susceptible		t127	Singletons			TET, SXT
HST 066	Tracheal Aspirate	Neg.	Resistant	V	t267	267			TET
HST 067	Tracheal Aspirate	Neg.	Susceptible		t127	Singletons	1	1	
HST 068	Pus	Pos.	Susceptible		t127	Singletons			CLI, ERY
HST 069	Sputum	Neg.	Susceptible		t315	306/002	361	361	CIP, ERY
HST 070	Wound	Neg.	Susceptible		t004	Singletons			
HST 071	Wound	Neg.	Susceptible		t004	Singletons			
HST 072	Pus	Pos.	Susceptible		t355	Singletons			
HST 073	Others	Neg.	Susceptible		t078	No founder	25	25	
HST 074	Others	Pos.	Susceptible		t3184	Singletons			CLI, ERY
HST 075	Eye	Neg.	Susceptible		t304	304/121	6	6	
HST 076	Tracheal Aspirate	Neg.	Resistant	V	t267	267			AUG, KF, OXA
HST 077	Sputum	Neg.	Resistant	III	t037	021	239	239	AUG, KF, CIP, CLI, ERY, OXA, TET, SXT
HST 078	Pus	Pos.	Resistant	IV	t6476	Singletons	80	80	AUG, KF, OXA
HST 079	Abscess	Pos.	Resistant	IV	t019	021			AUG, KF, OXA
HST 080	Tracheal Aspirate	Neg.	Susceptible		t132	Excluded	46	45	
HST 081	Tracheal Aspirate	Neg.	Susceptible		t774	084	15	15	
HST 082	Abscess	Pos.	Susceptible		t127	Singletons			CLI, ERY
HST 083	Wound	Pos.	Susceptible		t021	021			
HST 084	Abdominal	Neg.	Resistant	V	t9831	No founder	72	72	AUG, KF, OXA
HST 085	Wound	Neg.	Resistant	V	t9831	No founder			AUG, KF, OXA
HST 086	Blood	Neg.	Susceptible		t127	Singletons			
HST 087	Drainage	Pos.	Susceptible		t355	Singletons			
HST 088	Tracheal Aspirate	Neg.	Susceptible		t861	Singletons	508	45	
HST 089	Nose	Neg.	Susceptible		t091	No founder	7	7	
HST 090	Pus	Neg.	Susceptible		t349	No founder	1995	25	

HST 091	Bronchial Lavage	Pos.	Resistant	IV	t044	044	80	80	AUG, KF, OXA
HST 092	Tracheal Aspirate	Neg.	Susceptible		t002	306/002			CLI, ERY
HST 093	Pleural Tap	Neg.	Susceptible		t159	No founder			
HST 094	Pus	Neg.	Susceptible		t306	306/002			TET
HST 095	Others	Neg.	Susceptible		t166	No founder	34	30	TET
HST 096	Pus	Pos.	Susceptible		t021	021	30	30	
HST 097	Wound	Neg.	Susceptible		t084	084			
HST 098	Pus	Neg.	Susceptible		t1149	No founder	291	398	
HST 099	Others	Neg.	Susceptible		t6702	Singletons			
HST 100	Abdominal Fluid	Pos.	Resistant	IV	t044	044			AUG, KF, OXA
HST 101	Pus	Neg.	Susceptible		t1149	No founder			
HST 102	Tracheal Aspirate	Neg.	Susceptible		t267	267			
HST 103	Pus	Neg.	Susceptible		t315	306/002			CIP
HST 104	Pus	Pos.	Resistant	IVa	t690	186/786/690			KF, OXA, TET
HST 105	Pus	Pos.	Resistant	IVa	t690	186/786/690			AUG, KF, OXA, TET
HST 106	Ear	Neg.	Susceptible		t9830	Singletons			
HST 107	Others	Pos.	Resistant	IV	t044	044			AUG, KF, OXA, TET
HST 108	Others	Neg.	Susceptible		t166	No founder			
HST 109	Nose	Neg.	Susceptible		t091	No founder			
HST 110	Tracheal Aspirate	Neg.	Susceptible		t186	186/786/690	88	88	
HST 111	Sputum	Neg.	Susceptible		t377	Singletons	770	770	
HST 112	Fluid	Neg.	Susceptible		t012	021	30	30	
HST 113	Pus	Neg.	Susceptible		t4793	304/121			
HST 114	Sputum	Neg.	Susceptible		t377	Singletons			
HST 115	Others	Neg.	Susceptible		t659	No founder	720	121	CLI, ERY
HST 116	Pus	Neg.	Susceptible		t044	044	97	97	
HST 117	Pus	Pos.	Resistant	IV	t044	044			AUG, KF, OXA, TET
HST 118	Sputum	Neg.	Susceptible		t9841	Singletons			
HST 119	Abscess	Pos.	Resistant	IV	t131	044	80	80	AUG, KF, OXA
HST 120	Tracheal Aspirate	Neg.	Susceptible		t084	084	199	15	TET
HST 121	Pus	Pos.	Susceptible		t008	304/121	8	8	CIP
HST 122	Others	Neg.	Susceptible		t3468	No founder			

HST 123	Sputum	Neg.	Susceptible		t2467	304/121			
HST 124	Catheter	Neg.	Susceptible		t3468	No founder			
HST 125	Skin	Neg.	Susceptible		t021	021			
HST 126	Bronchial Lavage	Neg.	Susceptible		t9805	Singletons			
HST 127	Pus	Neg.	Resistant	IV	t9832	Singletons			AUG, KF, CIP, CLI, ERY, OXA
HST 128	Abscess	Pos.	Resistant	IV	t044	044			AUG, KF, OXA
HST 129	Tracheal Aspirate	Neg.	Susceptible		t267	267			
HST 130	Tracheal Aspirate	Neg.	Resistant	IV	t021	021	80	80	AIG, KF, OXA
HST 131	Sputum	Neg.	Susceptible		t021	021			
HST 132	Sputum	Neg.	Susceptible		t005	032	641	641	