Typing and Characterization of *Staphylococcus aureus* Isolated from Jordan

by

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ABSTRACT

Typing and Characterization of *Staphylococcus aureus* Isolated from Jordan

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Staphylococcus aureus remains an important pathogen worldwide. Methicillin-resistant *S. aureus* (MRSA) colonization have increased and caused serious invasive and life-threatening infections in young children. Nose and intestine are considered as important sites of colonization with *S. aureus*. Molecular epidemiological studies are essential to understand the genetic evolution, the pathogenesis and the dissemination of *S. aureus* in the region. The study aimed at the epidemiological typing of *S. aureus* in Jordan. 103 S. aureus, 41 MRSA and 62 Methicillin-susceptible *S. aureus* (MSSA), isolated from Jordanian children were characterized using different typing methods including *spa* typing, staphylococcal chromosomal cassette SCC*mec* typing, multilocus sequence typing (MLST) and Pulsed-Field Gel Electrophoresis (PFGE). The detection of Panton-Valentine Leukocidin (PVL) and the distribution of SCC*mec* type I-VII were performed by multiplex PCR assays. Isolates were 15% PVL positive, and were distributed

over 63 nose and 40 stool specimens. PVL were detected in 30% of MRSA and 1.6% of MSSA. 48 different spa types were identified and clustered into different groups with the most common spa types being t044, t065, t223 and t386. SCC*mec* typing showed the prevalence of types IV (a, b, c, d, g, and h) (53%), IVe (22%) and I (15%). 13 allelic profiles or STs were identified by MLST and the major type was ST80 (17%) followed by ST30 (13%), ST22 (13%) and ST15 (13%). ST80-MRSA-IV which is disseminated in Europe, Asia and the Middle East was the major clone in this study. 75% of MRSA harbor SCC*mec* IV which indicates that those infections in Jordanian children were most probably due to community-acquired MRSA (CA-MRSA). This study which showed great diversity between MRSA and MSSA is the first report characterizing *S. aureus* in Jordan using four molecular typing techniques. 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 the characterization of MRSA and MSSA in the region.

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GLOSSARY

bp: Base pair

BURP: Based upon repeat pattern

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

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

MLST: Multi-locus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

TSA: Tryptone Soy Agar

MSSA: Methicillin-sensitive *Staphylococcus aureus*

PCR: Polymerase chain reaction

PFGE: Pulse-field gel electrophoresis

PVL: Panton-Valentine leukocidin

S. aureus: *Staphylococcus aureus*

SCC: Staphylococcal chromosomal cassette

SCV: Small colony variant

***spa* CC:** *spa* clonal complex

spa: Gene for Staphylococcal Protein A

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..To them I dedicate this thesis

Chapter 1

INTRODUCTION

Staphylococcus aureus is the best known and most prominent staphylococcal species (Ng et al. 2009). It has been identified in the 1880s, in association with a wide range of serious and mild clinical manifestations (Deurenberg and Stobberingh 2008). *S. aureus* is a leading human pathogen implicated in many community- and hospital-acquired infections worldwide in all age groups (Lamaro-Cardoso et al. 2009). Colonization of different body sites, including the skin, nares, oral cavity and gastrointestinal tract is the step preceding infection caused by *S. aureus* (Todar 2008). Diseases ranging from superficial skin infections to severe endocarditis, pneumonia and bacteremia, may result from an infection (Strommenger et al. 2008). Several virulence factors contribute to the pathogenicity of *S. aureus* including Panton-Valentine Leukocidin (PVL), protein A, toxic shock syndrome toxin 1 (TSST-1), enterotoxins and exfoliative toxins. The induction of biofilm formation, by the adhesion of bacteria to a surface, followed by accumulation in multilayered cell clusters through intercellular adherence, is an additional vital infectious component (Izano at al. 2008).

Treatment of *S. aureus* infections is complicated due to the ability of this bacterial species to become resistant to antibiotics (Périchon and Courvalin 2009). In 1961, soon after the introduction of methicillin into clinical practice, methicillin-resistant *S. aureus* (MRSA) strains have emerged as an evolution of antimicrobial resistance (Hallin et al. 2007). This led to the appearance of hospital-adapted multiresistant clones that constitute

the major cause of nosocomial infections all over the world (Strommenger et al. 2008). MRSA emergence and dissemination are of global significance in both health care and community venues (Lamaro-Cardoso et al. 2009). MRSA has caused serious invasive and life-threatening infections in young children (Boyle-Vavra and Daum 2007). In addition, children may act as vectors helping *S. aureus* and specifically MRSA to spread in both community and hospital environments (Lamaro-Cardoso et al. 2009). Vancomycin is the drug used for therapy of infections due to MRSA (Périchon and Courvalin 2009). The emergence of resistance to vancomycin demands immediate characterization of isolates and identification of clonal spread within hospitals (Vindel et al. 2009).

Therefore efficient epidemiological typing methods are essential to monitor and limit the occurrence and spread of epidemic clones between hospitals; typing systems must facilitate the discrimination between unrelated isolates and the recognition of isolates that belong to the same clonal lineages.

The molecular approaches like *spa* typing, multilocus sequence typing (MLST), SCC*mec* typing, and pulsed-field gel electrophoresis (PFGE) can determine whether there is genetic relatedness between epidemiologically related isolates (Strommenger et al. 2008).

In this study we report on the typing and molecular characterization of *S. aureus* isolated from Jordan. The samples were taken from Jordan University hospital from children (nose and stool specimen) in 2008. PFGE, MLST, *spa* typing, and SSC*mec* typing, were the molecular typing techniques used for *S. aureus* characterization. The objectives of this study were (i) the molecular characterization of MSSA and MRSA isolates in

Jordan, (ii) to determine the diversity of *S. aureus* isolates associated with infections in Jordanian children (PVL, *mecA* genes, *spa* types, clonal complexes (CCs) and ST types), (iii) epidemiological typing of *S. aureus* using different molecular approaches, (iv) to ascertain a comparative analysis with other correlated studies in the region, especially with those from Lebanon.

Chapter 2

LITERATURE REVIEW

2.1 Overview of *Staphylococcus aureus*

Staphylococcus aureus is a gram positive facultative pathogenic bacterium causing a wide range of diseases from minor skin and soft tissue infections to life-threatening diseases (Stommenger et al. 2008). *S. aureus* is a spherical, non-flagellated bacterium (Kaito and Sekimizu 2007), 0.5 to 1.0 μm diameter, which on microscopic examination appears in short chains, pairs or grapelike structure, and as golden yellow colony when cultured on agar. The bacterium is catalase-positive and oxidase-negative, and its optimal growth temperature ranges from 15 to 45°C (Todar 2008). It is recognized as one of the most prevailing pathogen implicated in many community-acquired (CA) and hospital-acquired (HA) infections (Boyle-Vavra and Daum 2007). *S. aureus* strains can be distinguished by specific surface factors, its growth properties, the production of coagulase enzymes, and its ability to form a clot in the presence of fibrinogen (Todar 2008).

Protein A, PVL, TSST-1, enterotoxins and exfoliative toxins are major virulence factors contributing to the pathogenicity of *S. aureus* (Izano at al. 2008). Methicillin-resistant *S. aureus* (MRSA), due to drug resistance evolution, has become one of the most prevalent nosocomial pathogen all through the world (Reischauer et al. 2010). Hence staphylococci are major human pathogens that cause a variety of diseases ranging from skin and tissue infections to toxin-mediated disease, bacteremia and pneumonia (Holtfreter et al. 2007). Other clinical symptoms can result from an infection

like endocarditis, deep abscess formation and even death (Schouls et al. 2009).

2.2 Occurrence

The burden of *S. aureus* infections has been shown to be closely related to the susceptibly of the bacterium to antibiotics (Blanc et al. 2007). In 1940's, before the use of antibiotics, the morbidity rate due to *Staphylococcus aureus* infections was around 80% (Deurenberg and Stobberingh 2008). The use of penicillin did not start until 1940. After 10 years, in 1950, penicillinase-producing *S. aureus* became a therapeutic problem in hospitals. In 1961, soon after the introduction of methicillin drug, the first MRSA was isolated (Hallin et al. 2007). After its appearance, MRSA became increasingly endemic in many hospitals worldwide (Blanc et al. 2007). Several studies have revealed that MRSA emergence is related to susceptible background after the possessing of a staphylococcal chromosomal cassette (SCC*mec*) harboring the *mecA* gene (Donnio et al. 2007).

Today, *S. aureus* remains a leading cause of health care associated infections as well as community-acquired (CA) infections (Tenover et al. 2005). The study done by Levy and Marshall in 2004 reported that more than 95% of all *S. aureus* isolates are resistant to penicillin, and 40 to 60% of clinical isolates in the USA and the UK exhibit methicillin resistance. This shows the remarkable increase in the proportion of MRSA infections in the last two decades (Ho et al. 2009), with mortalities of up to 33% for MRSA infections and 16% with bacteremia caused by *S. aureus* (Wolk et al. 2009). Accordingly, the identification of the factors that influence the spread of *S. aureus* in hospitals is of great concern to prevent nosocomial infections,

where MRSA still a problem associated with high morbidity and mortality rate in hospitals (Donnio et al. 2007).

2.3 Pathogenesis of *Staphylococcus aureus*

S. aureus causes a wide range of diseases that are attained by many virulence factors contributing to the pathogenicity of the organism. These virulence factors include proteins, toxins and adhesins factors that are involved in initiating a series of immune reactions in the host (Kuroda et al. 2007). Surface proteins promote colonization, invasins (leukocidin, kinases, hyaluronidases) promote bacterial spread in tissues and surface factors inhibit engulfment (Todar 2008). Additionally, *S. aureus* produces collagen, fibrinogen, fibronectin-binding proteins (Chi et al. 2010), and has biochemical properties enhancing its survival in phagocytes, and superantigens that lead to the symptoms of septic shock. The expression of the virulence factors may occur either during the exponential or the stationary growth of the bacterium (Seybold et al. 2006). On the other hand, the pathogenicity of the bacterium is correlated to a group of toxins including staphylococcal enterotoxins, toxic shock syndrome toxin 1 (TSST-1), Panton-Valentine Leukocidin (PVL), epidermolysins A and B, leukotoxins, exofoliative toxins and others (Baba-Moussa et al. 2008).

The superantigen toxin TSST-1, which is encoded by *tsst-1* gene, is produced by some *S. aureus* isolates and is the major cause of toxic shock syndrome, staphylococcal scarlet fever and neonatal shock-like exanthematous diseases (Kikuchi et al. 2003). The nonspecific activation of 20 to 25% of T-cell is due to the ability of the superantigen to cross-link the class II major histocompatibility complex of antigen-presenting cells and T-cell receptor (TCR) b-chain variable regions leading to the production of IL-

4, IL-5, IL-13 and others, which in turn induces IgE production and inflammation (Patou et al. 2008).

PVL another important factor enhancing pathogenesis is a member of the family of "biocomponent synergohymenotropic toxins" causing the lysis of host cell membranes. The two proteins are encoded by two genes, *lukF*-PV and *lukS*-PV and are co-transcribed together (Dinges et al. 2000). The release of inflammatory mediators such as IL-8, leukotriene B4 and histamine from macrophages and monocytes is induced by PVL (Lina et al. 1999).

On the other hand, protein A is a conserved surface protein and a common virulence factor encoded by the genome of all *S. aureus* isolates. Protein A could be either secreted or membrane-associated, and interrelates with a variety of human and animal immunoglobulins (Silverman and Goodyear 2006). It prevents phagocytosis by binding to the Fc-region of IgG and causing vast inflammatory responses in human airways and corneal epithelial cells (Gómez et al. 2004) through eliciting T cell-independent B cell proliferation (Bekeredjian-Ding et al. 2007).

2.4 Resistance to Methicillin

In 1959, methicillin, a penicillinase-resistant penicillin was introduced for the treatment of penicillin-resistant *S. aureus* infections (Enright et al. 2002). MRSA is now a common pathogen associated with nosocomial and community-acquired infections (Lulitanond et al. 2009). First reports were recovered from the United Kingdom, and soon after from Japan and Australia (Shopsin et al., 2001). MRSA carriage plays an essential role in the spreading of the bacterium in health care facilities and the community (Mollema et al. 2010). Colonization by asymptomatic community-acquired MRSA (CA-MRSA) has been documented in healthy children visiting child

health care centers and outpatient clinics of childrens' hospitals (Huang et al. 2007). Treatment of MRSA infections is more difficult and tends to have pourer outcome, compared to methicillin-susceptible *S. aureus* (MSSA). Efficient epidemiological studies of MRSA isolates are essential for preventing their spreading, determining the clinical spectrum of MRSA diseases, and optimizing treatment (Dauwalder et al. 2008).

The resistance to methicillin is due to β-lactamase enzyme, which is responsible for the hydrolytic cleavage of the β-lactam ring present in penicillin. Four types of penicillin-binding proteins (PBPs 1-4) are produced by *S. aureus*. These membrane-bound transpeptidase proteins control the final stages of cell wall biosynthesis. The β-lactam antibiotics have a main role in disrupting the activity of PBPs (Murphy et al. 2008). MRSA contains a 21 kb gene called *mecA* (Deurenberg and Stobberingh 2008) located on a mobile genetic DNA fragment the staphylococcal cassette chromosome (SCC*mec*) (Nubel et al., 2008). Depending on the *mecA* gene complex and *ccr* gene complex they carry, SCC*mec* elements are classified into eight major variants (type I to VIII) (Zhang et al. 2009). Their sizes range from 20.9 to 66.9 Kb (Deurenberg and Stobberingh 2008), and they are classified into subtypes according to their junkyard region DNA fragment (Tokajian et al. 2010). The *J* (junkyard) regions (*J1*, *J2*, and *J3*) separate the *mec* and *ccr* gene complexes and display antibiotic resistant properties (Zhang et al. 2009). Thus the overall structure of the SSC*mec* is *J1-ccr-J2-mec-J3* (Milheirico et al. 2007). MRSA carriage plays an essential role in the spreading of the bacterium in health care facilities and the community (Mollema et al. 2010). Colonization by asymptomatic community-acquired MRSA (CA-MRSA) has been documented in healthy children visiting child health care centers and outpatient clinics of childrens' hospitals (Huang et al.

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2.5 Molecular typing of *Staphylococcus aureus*

Understanding the pathogen emergence, distribution and route of spreading is a primary step for the identification of the epidemiological and genetic correlations between the origins and types of bacterial strains (Deurenberg and Stobberingh 2008). Because *S. aureus* remains a major cause of health care- and community-associated (CA) infections, accurate typing methods are essential to develop cost-effective intervention and prevention strategies (Conceiçáo et al. 2009). These typing methods are important tools for the characterization and discrimination of isolates to determine the epidemiology of the infectious diseases based on the genotypic or the phenotypic characteristics of the isolates (Faria et al. 2008). This can be used for establishing clonal relationships between strains and tracing the geographic spreading of bacterial clones. Today, molecular methods are more useful to classify bacterial isolates than phenotypic methods because they provide great discriminatory power (Faria et al. 2008). The most common techniques used for typing staphylococci include pulsed-field gel electrophoresis (PEGE), multilocus sequence typing (MLST), *spa* typing and SCC*mec* typing. MLST and PFGE are the most reliable typing method with *S. aureus* (Aires de Sousa and De Lencastre 2004).Purpose, advantages and disadvantages differ between the 4 methods, but due to the progress in large scale sequencing methodologies, ease of data transfer and excellent

comparability of results, DNA sequence-based typing techniques are still the ones being most frequently used (Faria et al. 2008).

2.5.1 Pulsed-field gel electrophoresis (PFGE)

This technique is considered the gold standard for typing *S. aureus* (Tenover et al. 2008). It was developed by Schwarz and Cantor in 1984. PFGE which is a band based typing technique relies on macrorestriction of the genome (Tokajian et al. 2010). The basis of PFGE typing is the digestion of the bacterial chromosomal DNA with restriction enzyme that recognizes specific sites along the chromosome, generating large fragments of DNA. DNA fragments are separated by agarose gel electrophoresis in an alternating voltage gradient electric field (Trindade et al. 2003). Among tested restriction endonucleases, *SmaI* has been shown to be the one with best performance. Identification of the bacterial strain is determined through different migration profiles produced by the analysis of the banding pattern of the *SmaI*-digested DNA by specific software (Narukawa et al. 2009). The orientation of the electric field across the gel, in PEGE, is periodically changed allowing effective separation of DNA fragments according to size. Strains that differ by less than 3 bands are considered to belong to the same PFGE type, whereas strains that differ by more than 6 bands are considered to be unrelated (Tinelli et al. 2009). This method having high discriminatory accuracy and sensitivity, is time-consuming (several days) and expensive (Conceiçáo et al. 2009). Although PFGE is the most widely used method for MRSA typing, its use is limited by problems of interpretation, interlaboratory comparative results exchange, high costs and low throughput (Ruppitsch et al. 2006). However, PFGE has been shown to be an important discriminatory and sensitive technique in micro-(short term or local) and

macro-(long term, national, continental) epidemiological settings (Hallin et al. 2007).

2.5.2 Multilocus sequence typing (MLST)

The application of MLST for the characterization and typing of MSSA and MRSA was first described by Enright et al. in 2000. MLST was developed to scan housekeeping genes of the pathogen. Through time these conserved loci accumulate changes slowly, which reflect the evolutionary pattern of the pathogen (Cooper and Feil 2004). The development of MLST by Maiden et al. in 1998 as a tool targeting these conserved loci was to supply accurate portable data about the history and evolution of bacterial pathogen (Urwin and Maiden 2003). Typing of *S. aureus* with MLST to study its evolutionary history is applied by sequencing variable regions of 500 bp fragments of seven housekeeping genes which 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 the resulting seven alleles at each of the housekeeping genes results in the sequence type (ST) (Vivoni et al. 2005). Data analysis and the classification of strains into clonal complexes (CCs) are performed after sequencing, with the ancestral CC being the strain with the highest number of single locus variants (SLVs) (Cooper and Feil 2004). The classification of isolates into particular clonal lineages is enabled by the implementation of software algorithms such as enhanced Based Upon Related ST (eBURST) and Based Upon Repeat Patterns (BURP) to group the related sequence types (Mellmann et al. 2008). MLST delimits *S. aureus* into small number of spread and discrete CCs, and has proved to be a very successful epidemiological tool (Ruimy et al. 2009). MLST is unsuitable for

local outbreak investigations and has low discriminatory power (Cai et al. 2007). It is a laborious and expensive technique but it is easy to perform and very efficient in phylogenetic studies, allowing interlaboratory data exchange through a public database available on the internet (Te Witt et al. 2009). The combination of MLST with SCC*mec* typing has been used to define clonal types of MRSA (Conceiçáo et al. 2009).

2.5.3 spa typing

spa typing is a molecular technique that can determine the sequence variation of the polymorphic region X of the *S. aureus* protein A (*Spa* locus) (Khandavilli et al. 2008). Protein A is a cell wall component of *S. aureus* encoded by the *spa* gene. The polymorphic region, the region X, of the *spa* gene consists of a variable number of 21 to 27 bp repeats and it is located upstream of a region encoding the cell wall-binding sequence (Narukawa et al. 2009). Mutations, deletions and duplications lead to the variation in number and sequence of the repeats in this region (Kahl et al. 2005). These repeats create a profile used to cluster *S. aureus* isolates and to establish an internet database accessible by laboratories (Schouls et al. 2009).

spa typing is more frequently used than other methods as a single locus sequence typing for *S. aureus* (Conceiçáo et al. 2009). *spa* typing is a rapid, discriminatory, inexpensive and objective because it is easy to perform, accurate and reproducible (Strommenger et al. 2008). It is considered as important genotyping technique for epidemiological investigations (Chmelnitsky et al. 2008), and compared to PFGE, it has many advantages in terms of interpretation, speed and interlaboratory results exchange (Ruppitsch et al. 2006). Moreover, the availability of software algorithms, in order to group the related sequence types, enables the classification of *S.*

aureus isolates into particular clonal lineages (Strommenger et al. 2008). Clustering of *spa* types is performed by BURP (based Upon Repeat Patterns) grouping analysis (Mellmann et al. 2008). The interpretation of BURP clustering has allowed the use of Ridom Staph Type software (RIDOM GmbH, Wurzburg, Germany) (Deurenberg and Stobberingh 2008).

2.5.4 SCC*mec* typing

The identification of the mobile genetic element responsible for methicillin resistance (SCC*mec*) is required to characterize MRSA lineages along their genetic background (Chmelnitsky et al. 2008). SCC*mec* typing is considered as the reference method to define MRSA clones (Vindel et al. 2009) and is performed by multiplex PCR using 10 primers to identify the SCC*mec* type of MRSA isolates. The reference method to define MRSA clones. It is based on the identification of the mobile genetic element responsible for methicillin resistance (SCC*mec*). Five classes of *mec* gene complexes exist designated as A, B, C_1, C_2 and D, and five allotypes 1, 2, 3, 4 and 5 arranged in several combinations to form different SCC*mec* types (Oliveira et al. 2006). Eight major variants of SCC*mec* elements (type I to VIII) are recognized till now (Zhang et al. 2009). Their size range from 20.9 to 66.9 Kb. Depending on the *mecA* gene and *ccr* gene complexes, SCC*mec* elements are classified into subtypes according to their junkyard region DNA fragments. Among the eight SCC*mec* types, the types I, II and III have been associated with hospital-acquired infections, while types IV and V are associated with community-acquired infections (Deurenberg and Stobberingh 2008). Several studies have shown that the combination of SCC*mec* type and MLST type defined as the "clonal type" is internationally used for MRSA clones nomenclature (Hallin et al. 2007).

Chapter 3

MATERIALS AND METHODS

3.1 Clinical isolates

A total of 103 *S. aureus* isolates obtained from children clinical specimens in the period between August 2008 and October 2008 were kindly provided by Dr. Asem Shehabi from Jordan University hospital in Amman-Jordan. The isolates were recovered from 63 nose and 40 stool specimens from children aged 1 to 12 months. Isolates were both MRSA and MSSA. The samples were streaked on Tryptone Soy Agar (TSA) and stored in Cryobanks at -20°C and -80°C. The isolates were designated by JS 1-103.

3.2 DNA Extraction

Isolates were grown overnight on TSA at 37 °C. DNA extraction was done using InstaGene matrix solution (BIO-RAD, München, Germany) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the 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 Perkin Elmer GeneAmp 9700 thermal cycler (PerkinElmer, Wellesly, Massachusetts). The 16S rRNA gene served as an internal control was amplified using gene-sequence-specific primers: Staph756F (5'-AAC TCT GTT ATT AGG GAA GAA CA-3') and Staph750R (5'-CCA CCT TCC TCC GGT TTG TCA CC-3'). The *lukS-PV* genes were amplified using Luk-

PV-1(5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3') and Luk-PV2 (5'-GCA TCA AGT GTA TTG GAT AGC AAA AGC-3') primers, and the *mecA* gene was amplified using MecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A-3') and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A-3') primers. 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 75 s 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 5-10µl of ethidium bromide staining using 1.5% agarose gel with 1x Tris-acetic acid-EDTA (TAE) buffer at 100V.

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. The amplification employed 10pmol of the *spa*-1-for (5'- TAA AGA CGA TCC TTC GGT GAG C -3') and 10pmol 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 5-10µl 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 left at 20°C for infinity.

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 1.0µl of premix from the kit, 1.5µl TrisHCl/$MgCl_2$ buffer (400mM Tris-HCl; 10mM $MgCl_2$), 10pmol of sequencing primer, and 2µl of the cleaned PCR product in a total volume of 10µl. The same primers used in the PCR were used for sequencing with an annealing temperature of 60°C. All sequencing reactions were performed with 25 cycles of denaturation (96°C, 10s), and extension (60°C, 4min).

3.4.4 DNA Precipitation and Loading

The sequencing products were purified using the Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ). The samples were dried in a vacuum centrifuge, and then 20µl of HiDi formamide were added (Applied Biosystems) as loading media for sequencing electrophoresis on an ABI 3100 Avant Genetic Analyzer. The software Ridom StaphTypeTM (Ridom GmbH, Würzburg, Germany) was used for *spa* sequence analysis.

3.5 MLST

For amplification of the 7 house-keeping genes, a PCR was performed in a total volume of 20 µl using the 7 housekeeping genes primers for amplification. The set of primers of all the seven housekeeping genes is arc-F (5'-TTG ATT CAC CAG CGC GTA TTG TC-3'), arc-R (5'-AGG TAT CTG CTT CAA TCA GCG-3'), aroE-F (5'-ATC GGA AAT CCT ATT TCA CAT TC-3'), aroE-R (5'-GGT GTT GTA TTA ATA ACG ATA TC-3'), glpF-F (5'-CTA GGA ACT GCA ATC TTA ATC C-3'), glpF-R (5'-TGG TAA AAT CGC ATG TGC AAT TC-3'), gmk-F (5'-ATC GTT TTA TCA GGA CCA TCT G-3'), gmk-R (5'-TCA TTA ACT ACA ACG TAA TCG TA-3'), pta-F (5'-GTT AAA ATC GTA TTA CCT GAA GG-3'), pta-R (5'-GAC CCT TTT GTT GAA AAG CTT AA-3'), tpi-F (5'-TCG TTC ATT CTG AAC GTC GTG AA-3'), tpi-R (5'-TTT GCA CCT TCT AAC AAT TGT AC-3'), yqiL-F (5'-CAG CAT ACA CAC CTA TTG GC-3'), yqiL-R(5'-CGT TGA GGA ATC GAT ACT GGA AC-3'). The reaction contained 2 µl DNA, 200 µM dNTPs, 10 pmol of each primer, 5 µl of 10-fold concentrated PCR Buffer II (Applied Biosystems), $MgCl_2$ 1.5 mM, and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). Thermal cycling reactions consisted of an initial denaturation (12 min at 95°C) followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension (60 s at 72°C), with a single final extension (10 min 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.

3.6 PFGE

13 isolates representing different MLST (STs) types were typed using PFGE technique to reveal a banding pattern on the gel. The protocol was adapted from Cookson et al. (2007) with slight modifications in it. Two to three colonies were inoculated in a 5 mL Brain Heart Infusion (BHI) Broth overnight. Then 1ml of each cell suspension is centrifuged at 15,000 xg for 1min and the pellet was resuspended in a 0.5mL of TEN buffer (Tris-EDTA-NaCl). After 1min centrifugation at 15,000 xg for 1min, the pellet was then resuspended in 0.5ml of EC (Tris-HCl, NaCl, di-Na EDTA, Brij-58, Na Deoxycholate, Sarkosyl) buffer and hold at 55°C. To each microfuge tube a 0.5ml low melting agarose and 25µl of recombinant lysostaphin (2mg/ml) were added and quickly mixed. The inserts were casted and left to harden and placed in 4ml EC buffer and 10-15µl RNase in a tube to be incubated at 37°C overnight. The next day the EC buffer was replaced with 4ml TE buffer (10mM Tris-HCl-1mM EDTA) 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-original) and washed for 30min at 4°C. The process was repeated four times with the new buffer prepared and kept overnight at 4°C. The buffer was discarded and replaced with a fresh 200µl 1x buffer A (Roche-original) and 1µl enzyme *SmaI* and incubated for 4 hours at 25°C in a thermoblock. The slice was then washed twice with 200µL 0.1x buffer A (Roche original) for 15min each time to finally stop the reaction with 20µl of stop mix, and stored at 4°C for 10min before gel running. The bands were resolved with a 1% agarose gel in 0.5x TBE and run in the electrophoresis apparatus (CHEF-DRIII System, BIO-RAD) using 0.5x TBE with an initial time 5sec and a final time 50sec at

14°C for 23.5 hours. The bands were then visualized under UV (SYNGENE-G:box) after staining with EtBr.

3.7 SCC*mec* typing

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) (Table 1). The protocol was adapted from Milheiric'o et al. (2007) with slight modifications where a multiplex PCR was performed using 10 primers to identify the banding pattern of the MRSA isolates and classify them into the corresponding SCC*mec* type. The optimal cycling conditions were the following: 94°C for 4 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 4 min. Each PCR mixture, in a final volume of 50 µl, contained 5 ng of chromosomal template; 1x PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems); 40 µM (each) deoxynucleoside triphosphate (MBI Fermentas, Hanover, MD); 0.2 µM primers kdp F1 and kdp R1; 0.4 µM primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1 F, and SCC*mec* V J1 R; 0.8 µM primers mecI P2, mecI P3, dcs F2, dcs R1, mecA P4, mecA P7, ccrB2 F2, ccrB2 R2, ccrC F2, and ccrC R2; and 1.25 U of Amplitaq DNA polymerase (Applied Biosystems). The PCR products (10 µl) were resolved in a 3% Seakem (Cambrex, Rockland, ME) agarose gel in 0.5% Tris-borate-EDTA buffer (Bio-Rad, Hercules, CA) at 4 V/cm for 2.5 h and were visualized with ethidium bromide. The set of primers used for SCC*mec* PCR is listed in Table 2.

Table 1.Reference strains used to optimize the conditions for the multiplex PCR. (Ito et al. 2001 ; Zhang et al. 2005).

Reference Strains	SCC*mec* type [a]	*mec* complex [b]	*ccr* complex [c]
NCTC 10442	I	Class B	Type 1
N315	II	Class A	Type 2
85/2082	III	Class A	Type 3
JCSC 4744	IV a	Class B	Type 2
JCSC 2172	IV b	Class B	Type 2
JCSC 4788	IV c	Class B	Type 2
WIS	V	Class C	Type 5

[a] **Subtypes of SCC*mec* IV** differ based on the junkyard (J) region of DNA. **b Class A mec:** IS431-mecA-mecR1-mec1; **Class B mec:** IS431-mecA-ΔmecR1-IS1272; **Class C mec:** IS431-mecA-ΔmecR1-IS431. [c] **Type 1 *ccr*:** *ccrB1-ccrA1*; **type 2 *ccr* :** *ccrB2-ccrA2* ; **type 3 *ccr* :** *ccrB3-ccrA3* ; **type 5 *ccr*:** *ccrC*

Table 2. Primers used in the SCC*mec* multiplex PCR. (Milheiric'o et al. 2007)

Primer name	Primer sequence (5'→3')	Primer specificity (SCC*mec* type, region)	Amplicon size (bp)
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	I, J1 region	495
CIF2 R2	ATTTACCACAAGGACTACCAGC		
ccrC F2	GTACTCGTTACAATGTTTGG	V, *ccr* complex	449
ccrC R2	ATAATGGCTTCATGCTTACC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	III, J3 region	414
RIF5 R13	ATGGAGATGAATTACAAGGG		
SCCmec V J 1F	TTCTCCATTCTTGTTCATCC	V, J1 region	377
SCCmec V J1 R	AGAGACTACTGACTTAAGTGG		
dcs F2	CATCCTATGATAGCTTGGTC	I, II, IV, and VI, J3 region	342
dcs R1	CTAAATCATAGCCATGACCG		
ccrB2 F2	AGTTTCTCAGAATTCGAACG	II and IV, *ccr* complex	311
ccrB2 R2	CCGATATAGAAWGGGTTAGC		
kdp F1	AATCATCTGCCATTGGTGATGC	II, J1 region	284
kdp R1	CGAATGAAGTGAAAGAAAGTG G		
SCCmec III J1 F	CATTTGTGAAACACAGTACG	III, J1 region	243
SCCmec III J1 R	GTTATTGAGACTCCTAAAGC		
mecI P2	ATCAAGACTTGCATTCAGGC	II and III, *mec* complex	209
mecI P3	GCGGTTTCAATTCACTTGTC		
mecA P4	TCCAGATTACAACTTCACCAGG	Internal positive control	162
mecA P7	CCACTTCATATCTTGTAACG		

Chapter 4

RESULTS

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

The 103 isolates were identified by multiplex PCR of the 16S rRNA, *lukS-PV* and *mecA* to differentiate MRSA from MSSA and indicate whether each isolate is a PVL producer or not. Isolates tested had 3, 2 or 1 band/s. The first band corresponds to a 756bp size and refers to the partial amplification of the 16S rRNA gene. The second band lies between the 400 and 500bp and with its size being specifically 433bp, it is the amplification product of the PVL gene. The third band corresponds to the amplification product of the *mecA* gene and is of a size of 310bp band (Fig 1).

Fig 1. 1.5% agarose gel electrophoresis showing representative PCR products after 16S rRNA, PVL and *mecA* amplification. The first and the second lanes show DNA marker 100bp and 500bp (Fermentas) lanes 1-11 show PCR products of the three genes of different isolates. Lane 12 is the negative control.

Table 3 represents the results of the triplex PCR for all the 103 isolates, with 40% (41 samples) of the 103 isolates being MRSA and 60% (62 samples) being MSSA. 88 isolates were PVL negative (85%) and the remaining 15% (15 isolates) were PVL positive. Of the 15 PVL positive isolates, 14 were MRSA and the remaining 1 was MSSA and of the 88 PVL negative isolates, 27 were MRSA and 61 were MSSA.

Table 3. The distribution of isolates among MRSA and MSSA, PVL positive and PVL negative.

Methicillin	Number of samples (%)	PVL + (%)	PVL – (%)
Resistant (MRSA)	41 (40%)	14 (34%)	27 (66%)
Susceptible (MSSA)	62 (60%)	1 (2%)	61 (98%)
Total	103	15 (15%)	88 (85%)

4.2 *spa* typing

For all 103 isolates, the bands obtained after the *spa* gene amplification varied in size between 250bp and 600bp depending on the number of repeats within the gene (Fig 2). All 103 isolates were typeable. *Spa* sequence typing was essentially performed according to RIDOM protocol using RidomStaphType Version (1.5) software. The analysis revealed 48 different *spa* types with the most common being t044 representing 11% of the isolates. The other most common *spa* types included: t065, t386, t223, t330, t338, t701 representing 10%, 8%, 7%, 4%, 4% and 4% of the studied isolates, respectively (Fig 3).

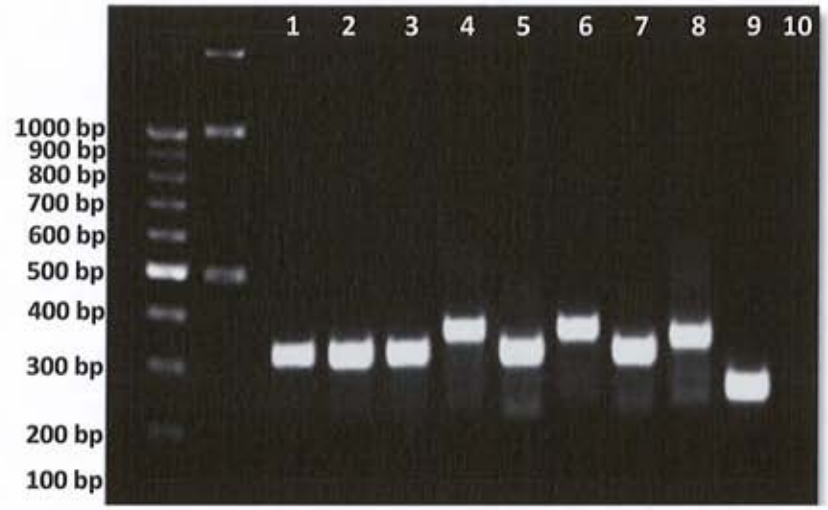

Figure 2. 1.5% gel electrophoresis showing PCR products after *spa* gene amplification. The first and the second lanes show DNA marker 100bp and 500bp (Ferrmentas); lanes 1-9 show PCR products of the amplified *spa* gene with sizes between 250 bp and 450 bp. Lane 10 is the negative control.

Within the MRSA the most common *spa* types were t044 (27%) and t386 (20%). The most common *spa* type within the MSSA was t065 (16%) (Fig 4). Using BURP algorithm, the 48 *spa* types were clustered so that each *spa* type belongs to one cluster and is related to at least one other *spa* type within the cluster (Fig 6). There were 8 *spa* CCs (clonal complexes), 8 singletons, 3 clusters with no founder, and 10 *spa* types were excluded (the algorithm only clusters the *spa* types with 5 repeats and more) (Fig 5).

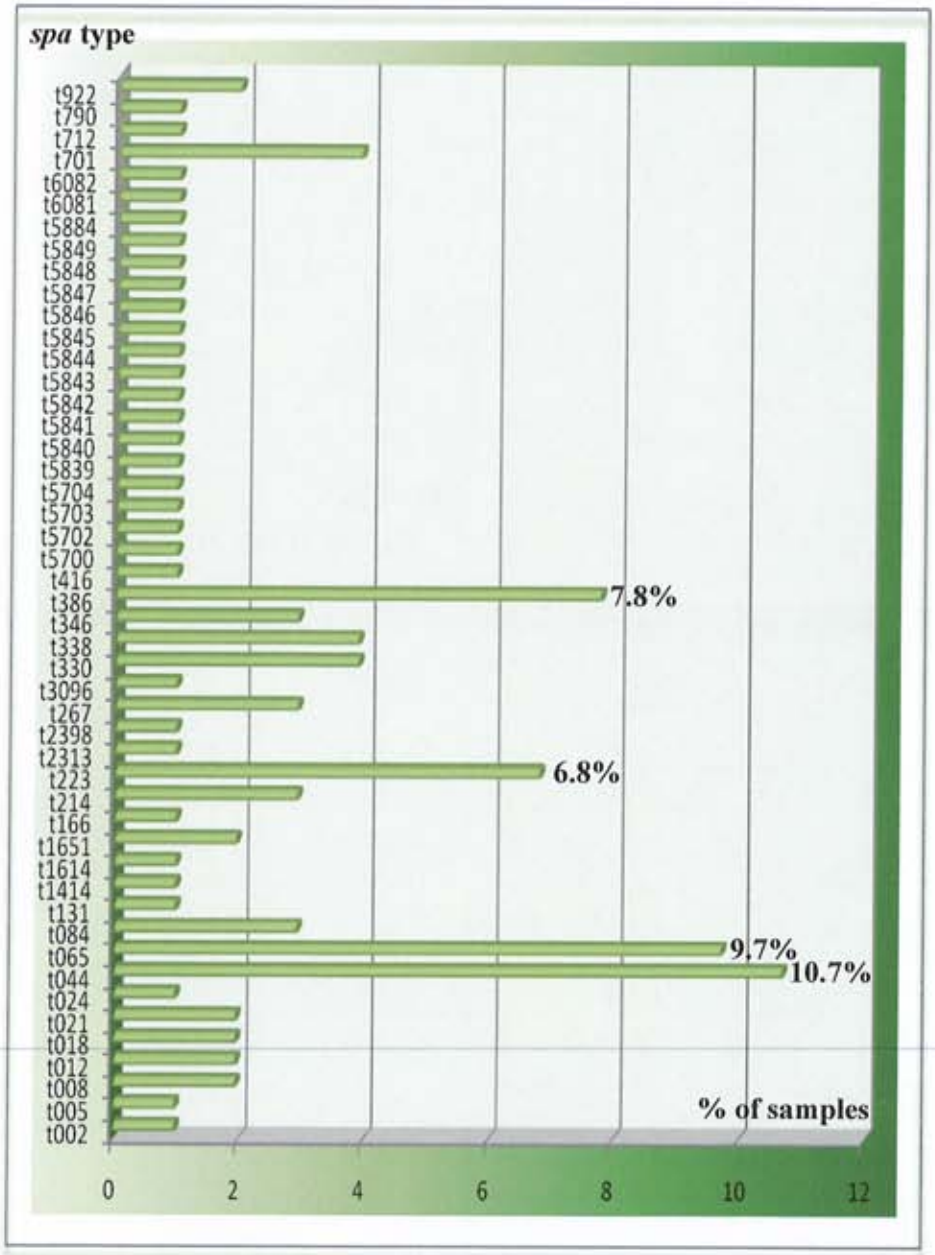

Fig 3. Percentage of the 48 *spa* types representing all the isolates undertaken in this study, where the predominated *spa* type was t044 (10.7%) followed by t065 (9.7%) and t386 (7.8%).

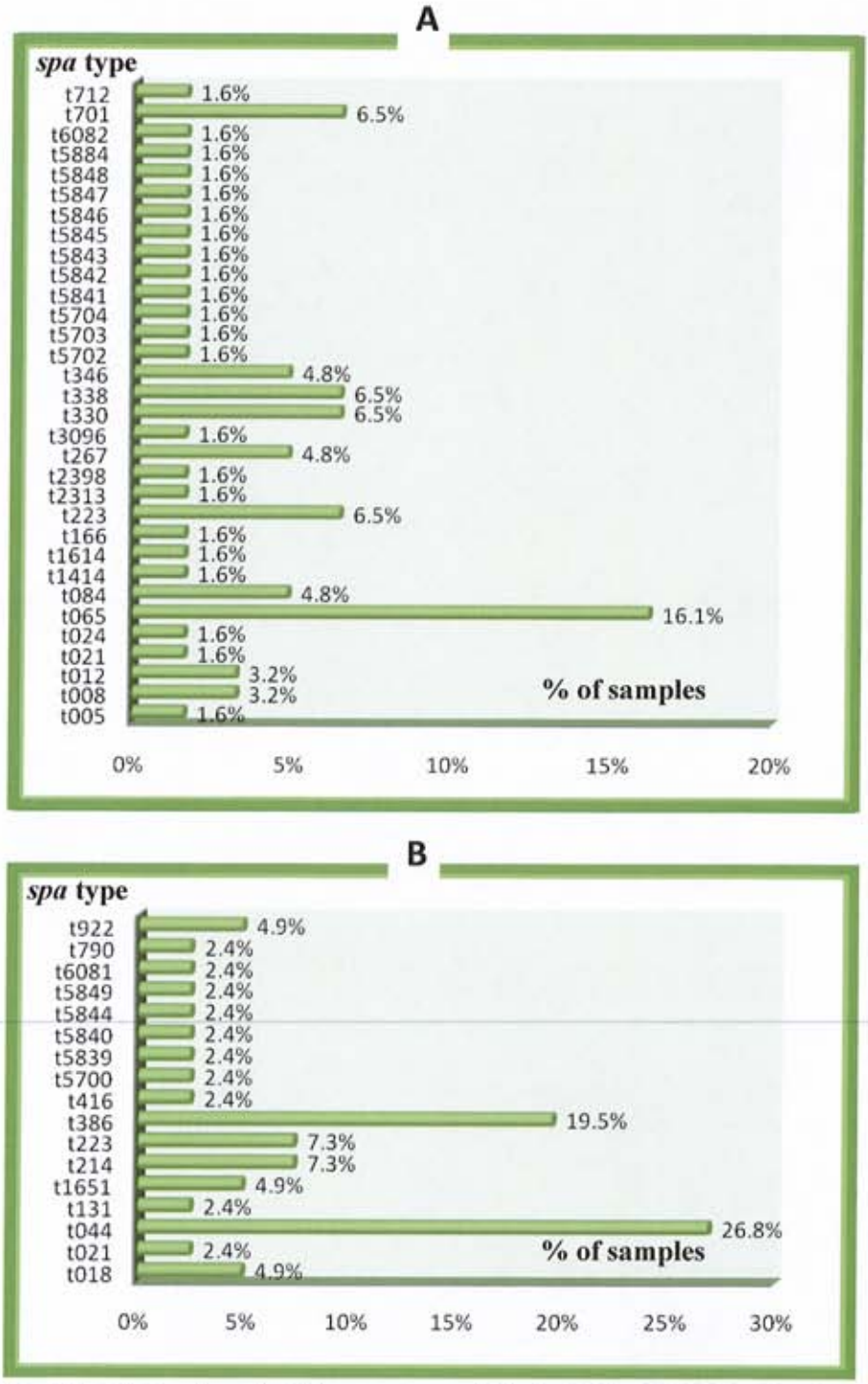

Fig 4. Distribution along the different *spa* types within A, MSSA; B, MRSA, where the most common *spa* types were t065 (16.1%) within MSSA, t044 (26.8%) and t386 (19.5%) within MRSA.

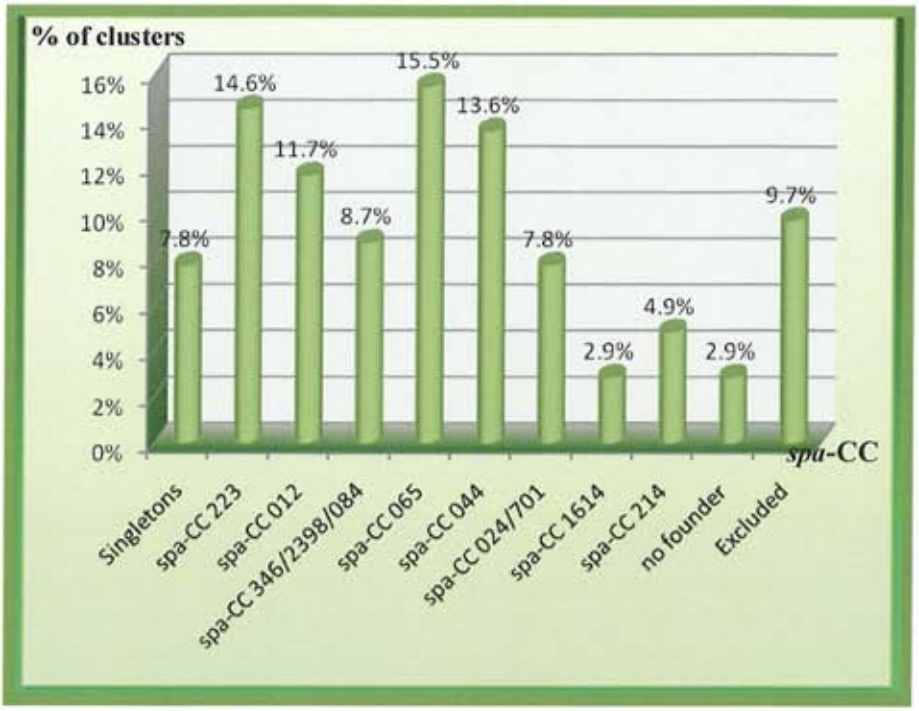

Fig 5. Clustering *spa* types by BURP showing the number of samples belonging to each *spa*-CC.

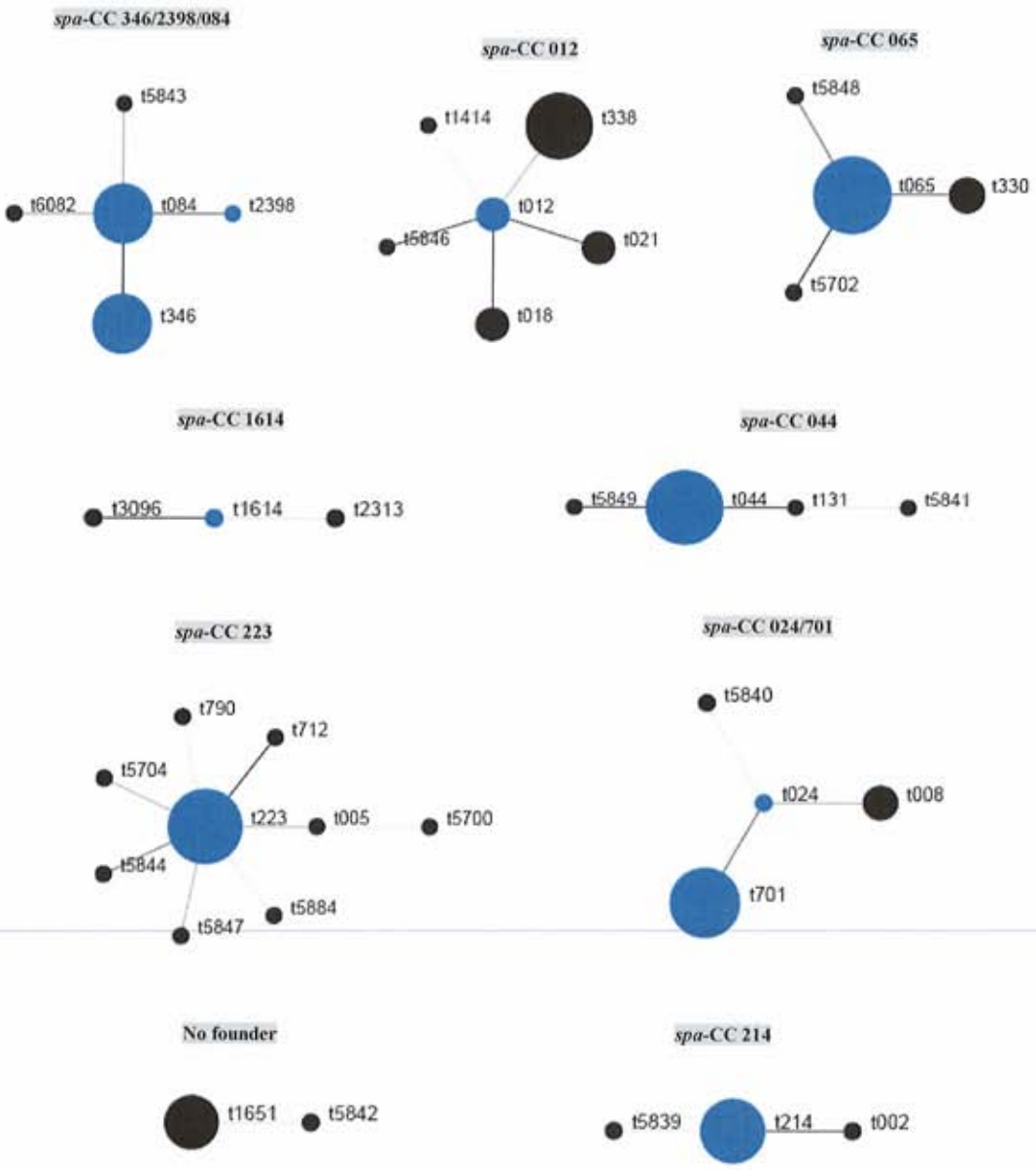

Fig 6. Population snapshot based on BURP analysis of 103 isolates. BURP grouping using default parameters resulted in 8 *spa* CCs, 8 singletons, and 10 excluded *spa* types . Each dot represents a unique *spa* type. The diameter of the a dot is proportional to the quantity of the corresponding *spa* type. Blue dots represent group founders, defined as the *spa* type with the highest founder score within a CC.

4.3 MLST

MLST analysis was done on 30 isolates representing most clonal complexes (CCs) among our studied strains, and including both MRSA and MSSA isolates. MLST analysis identified 13 distinct allelic profiles or STs among the isolates, with the majority of isolates belonging to ST80. Other STs included ST30, ST22, ST15, and ST5. ST80s were PVL positive except for one isolate of MSSA origin, which was PVL negative. All these STs and their allelic profiles and distribution among *spa* types and *spa* CCs are listed in Table 4.

Isolate	PVL[1]	*spa* type	*spa*-CC[2]	Allelic profile	MLST ST[3]
JS 22	-	t005	223	2-2-2-2-6-3-2	30
JS 55	-	t223	223	7-6-1-5-8-8-6	22
JS 15	-	t223	223	2-2-2-2-6-3-2	30
JS 47	-	t790	223	2-2-2-2-6-3-2	30
JS 52	-	t012	012	7-6-1-5-8-8-6	22
JS 01	-	t021	012	7-6-1-5-8-8-6	22
JS 70	-	t021	012	7-6-1-5-8-8-6	22
JS 54	-	t084	346/2398/084	13-13-1-1-12-11-13	15
JS 31	-	t346	346/2398/084	13-13-1-1-12-11-13	15
JS 32	-	t346	346/2398/084	13-13-1-1-12-11-13	15
JS 03	-	t065	065	10-14-8-6-14-3-2	46
JS 64	-	t330	065	10-14-8-6-14-3-2	46
JS 45	+	t044	044	1-3-1-14-11-51-10	80
JS 60	+	t044	044	1-3-1-14-11-51-10	80
JS 71	+	t131	044	1-3-1-14-11-51-10	80
JS 82	+	t5849	044	1-3-1-14-11-51-10	80
JS 20	-	t008	024/701	2-2-2-2-6-3-2	30
JS 56	-	t024	024/701	3-3-1-1-4-4-3	8
JS 17	-	t701	024/701	12-4-1-4-12-1-3	6
JS 10	-	t002	214	1-4-1-4-12-1-10	5
JS 77	-	t214	214	1-4-1-4-12-1-10	5
JS 18	-	t5839	214	1-4-1-4-12-1-10	5
JS 50	-	t1651	No founder	10-47-8-26-26-32-2	395
JS 85	-	t5842	No founder	8-2-2-2-6-3-2	34
JS 65	-	t166	Singletons	3-1-1-1-1-5-3	97
JS 61	-	t267	Singletons	1-1-1-1-1-1-1	1
JS 08	-	t922	Singletons	4-1-4-1-5-5-4	25
JS 84	-	t6081	Singletons	1-1-1-1-1-1-1	1
JS 07	-	t386	Excluded	1-3-1-14-11-51-10	80
JS 25	+	t5845	Excluded	10-47-8-26-26-32-2	395

1. Panton-Valentine leukocidin; 2. *spa* clonal complex; 3. Multilocus sequence typing sequence type

Table 4. Properties of the resulting STs with their allelic profiles and distribution among *spa* types and *spa* CCs.

4.4 PFGE

Representative of each of the 15 *spa* types selected from different ST types were genotyped by PFGE after *SmaI* enzyme digestion of chromosomal DNA. Unlike *spa* typing, PFGE showed higher ability to depict variations among isolates. Samples belonging to the same *spa* type t044 (Fig 7 and 8) had similar and/or different banding patterns (more than 3 bands difference according to McDougal et al. (2003) (Fig 9).

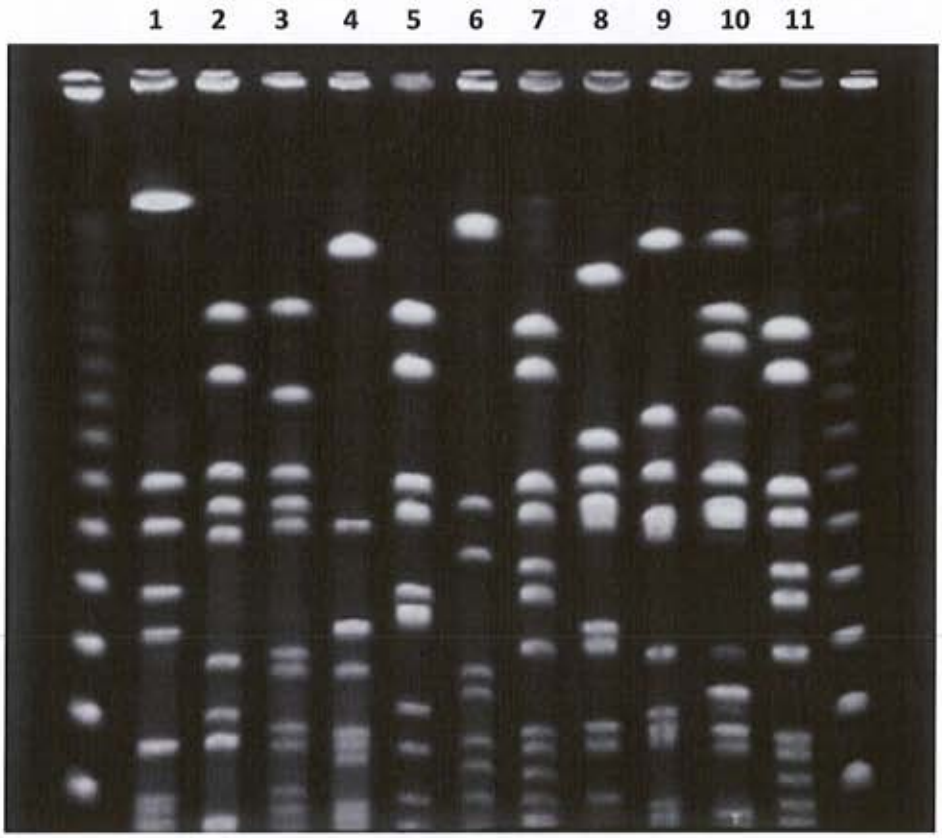

Fig 7. PFGE representing 11 samples of different *spa* types (lanes 1-11). The first and last lanes represent BioRad DNA marker.

Lanes 2 (spa type t002) and 3 (*spa* type t701) are of different *spa* types and show an almost identical banding pattern on the gel. Lanes 3 (*spa* type t701) and 8 (*spa* type t024) belong to different *spa* types within the same spa-CC, both PVL- and stool specimen and have similar banding pattern differing in two bands.

4.5 SCC*mec* typing

The SCC*mec* types among the MRSA isolates revealed predominance (53%) of subtype IV (a, b, c, d, g, h), followed by subtype IVe (22%) and type I (15%) (Fig 7, 8). SCC*mec* types I to III generate specific amplification patterns of three to five bands. SCC*mec* type IV generates an amplification pattern of three bands (*mecA, ccrB2*, and *dcs*) for subtypes a to d, g, and h; and subtypes IVe and IVf generate only two bands, since they are negative for the *dcs* locus. SCC*mec* type V also originates a pattern of three bands (*mecA, ccrC*, and J1). SCC*mec* type VI, which appears to be an "exotic" local variant, generates only two bands (*mecA* and *dcs*), and its assignment must be confirmed by *ccrAB* allotyping (*ccrAB4*). Note that SCC*mec* types IV and VI previously could not be discriminated since both were positive only for *mecA* and *dcs* (Milheiric'o et al. 2007).

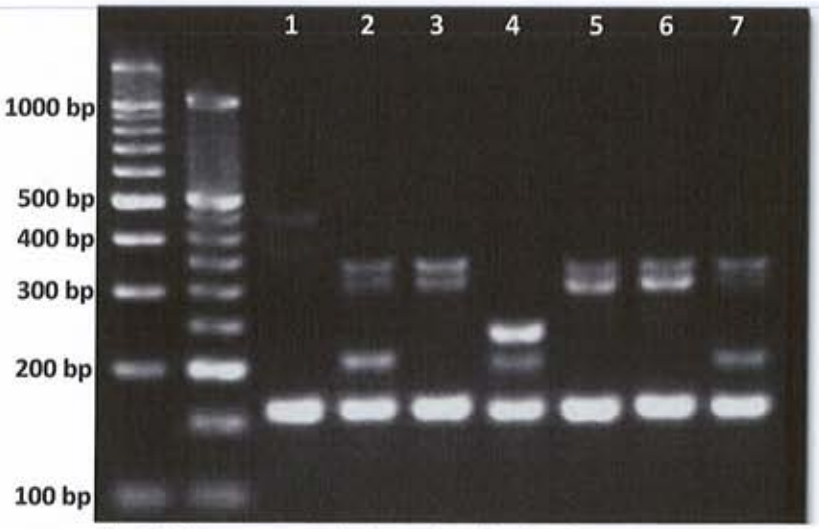

Fig 8. 3% agarose gel electrophoresis showing representative PCR products after SCC*mec* subtyping of 7 isolates. The first and the second lanes show DNA marker 100bp and 50bp (Ferrmentas). Lane 1: type V; lanes 2,7: type II; lanes 3,5,6: type IV a, b, c, g, h .

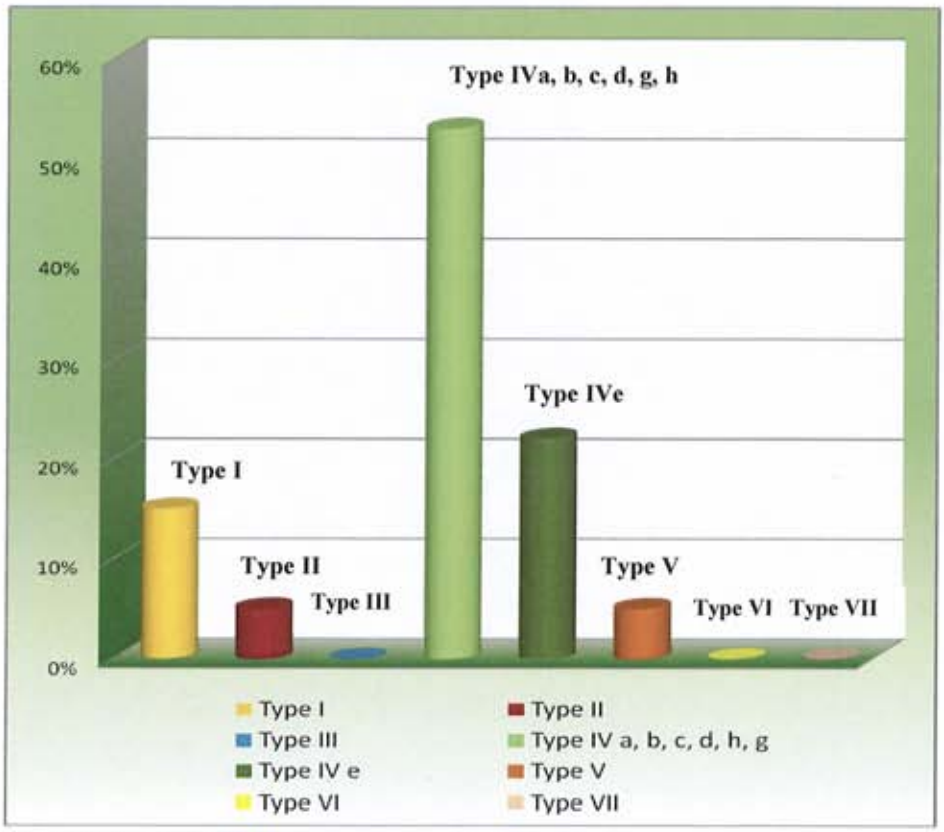

Fig 9. Distribution of SCC*mec* subtypes among MRSA isolates.

Chapter 5

DISCUSSION

S. aureus is an important pathogen associated with a wide range of serious and mild clinical manifestations (Deurenberg and Stobberingh 2008), with MRSA causing serious invasive and life-threatening infections in young children (Boyle-Vavra and Daum 2007). Many questions on the epidemiology of MRSA carriage and infections remain unanswered due to the spread and evolution of MRSA worldwide. Today, molecular typing techniques like *spa* typing, PFGE, MLST and SCC*mec* typing may shed light on the epidemiology and evolutionary relationships of *S. aureus* and specially MRSA (Chmelnitsky et al. 2008). Those methods can offer a high degree of typeability and reproducibility (Mellmann et al. 2008), especially with the implementation of software algorithms, such as BURP and eBURST. Having such softwares helped in the grouping of related sequence types, and enabled an easier access into the classification of isolates into particular clonal lineages.

Many reports have revealed that MRSA infections in children have increased (Eady and Cove 2003). Several factors have shown to make children as host more susceptible to MRSA than adults, i) children are more exposed to different environmental factors such as schools and daycare centers, which increase the risk factors for MRSA colonization, ii) children are treated using antimicrobial drugs different than the ones used with adults, which may vary the antimicrobial drug selection pressure, iii) differences in host defense makes children more vulnerable to encounter novel MRSA strains (Park et al. 2007). Other factors that make children susceptible to severe bacterial

infections include malnutrition, exposure to unsanitary conditions, and lack of breast feeding (Marcovici et al. 2005).

In this study MRSA and MSSA from Jordan were epidemiologically characterized by different molecular techniques to determine the diversity of *S. aureus* isolates in Jordanian children, and to ascertain a comparative analysis with other correlated studies in the region. 103 isolates (15% PVL positive and 85% PVL negative) were distributed over 63 nose and 40 stool specimens. Within the nose specimens 13% were PVL positive, while it was 17% for the stool. Nose and intestine are considered as important site of colonization with *S. aureus*. Although the anterior nares are considered to be the primary site of infections, Bhalla et al. (2007) revealed the important clinical implications of the intestinal tract colonization by *S. aureus*. In the same study Bhalla et al. (2007) found a coexistence of intestinal colonization with *S. aureus* in more than half of patients with vancomycin-resistant *Enterococcus* (VRE), which facilitates the emergence of *S. aureus* isolates resistant to vancomycin and mandates analyzing stool specimens. The data obtained from the Jordan university hospital revealed that all recovered isolates were susceptible to vancomycin, and our results showed that 41% (stool and nose) were resistant to methicillin with even distribution being detected within both types of specimens. The high percentage of MRSA was in concordance with Bhalla et al. (2007) findings with the percentage among stool and nose carriers being approximately 41%. Lo et al. (2010) reported that 7% (89 out of 1195 children) had nares cultures positive for MRSA and it was 30% among all *S. aureus* isolates studied. The dissemination of MRSA clones distinct from nosocomial MRSA isolates in healthy children was revealed by PFGE in the same study. Acton et al. (2009) revealed that MRSA colonized 1-2% of young children and newborns, while Lindberg et

al. (2004) showed that *S. aureus* was detected in 60% of stool specimens collected from 1 month-old-children with 90% of the strains matching to a parent skin strain.

spa typing in this study revealed 48 different types with the most commonly occurring ones being: t044, t065, t386 and t223. MRSA (n=41) were distributed over 17 *spa* types, as opposed to 33 *spa* types for the MSSA (n=62) isolates. This reflects a higher diversity in the genetic profile of MSSA compared to MRSA as reported also by Grundmann et al. (2010). Among the MRSA, 34 % were PVL positive, while it was 1.6% for the MSSA. This was in concordance with the higher prevalence of PVL positive MRSA isolates in Europe (Vindel et al. 2009), while it was 17% of MRSA nasal isolates in northern Taiwan (Lo et al. 2010). All the *spa* t004 (27% of MRSA) and *spa* t386 isolates (19% of MRSA) were MRSA, while all the *spa* t065 (16% of MSSA) were MSSA. However, *spa* type t223 was distributed among both MSSA (6%) and MRSA (7%). This agrees with the notion that MRSA was derived recently from a limited number of MSSA lineages by the acquisition of the SCC*mec* element (Enright et al. 2002). The high frequency of t044, especially within the MRSA, was in concordance with the results obtained by Tokajian et al. (2010) in Lebanon, where 38 % of the isolates were of *spa* t044 and MRSA. Vindel et al. (2009) additionally revealed that *spa* t044 was widely disseminated in Europe, which was again in harmony with our results.

Using BURP algorithm, the 48 *spa* types were clustered so that each was grouped into one cluster and was related to at least one other *spa* type within the cluster. *spa*-CC 065 (15%), *spa*-CC 223 (14%), *spa*-CC 044 (13%), *spa*-CC 012 (12%) were the most common clonal complexes having more than one *spa* type. Both MSSA and MRSA isolates were grouped under *spa*-CC

223, *spa*-CC 044, and *spa*-CC 012, while only MSSA within *spa*-CC 065. Isolates belonging to *spa*-CC 044 and *spa*-CC 012 were all PVL negative except for *spa* t044 (PVL positive), while those belonging to *spa*-CC 065 and *spa*-CC 012 had representatives of both.

Thirty isolates (including MRSA and MSSA) representing most clonal complexes (CCs) recovered in this study were typed using MLST. Thirteen distinct allelic profile or STs were identified with the majority (17%) of the isolates belonging to ST80, which again agreed with results obtained from Lebanon (Tokajian et al. 2010). Other major STs were ST30 (13%), ST22 (13%), and ST15 (13%). ST80 is known to be common in Asia, Europe, and the Middle East, ST30 in Europe, Australia and South America, ST22 in parts of Europe (Robinson and Enright 2004). All ST22 and ST15 isolates were PVL negative while ST80 and ST30 included both PVL positive and negative.

SCC*mec* typing is considered as the reference method to define MRSA clones (Vindel et al. 2009), thus it is required to characterize MRSA lineages along their genetic background (Chmelnitsky et al. 2008). Hospital-acquired MRSA (HA-MRSA) isolates are mainly negative for PVL, resistant to multiple antibiotics and carry SCC*mec* types I, II, III and rarely IV. On the other hand CA-MRSA isolates are frequently positive for PVL, susceptible to the majority of non-β-lactam antibiotics and harbor mainly SCC*mec* IV or V. The SCC*mec* IV along with the PVL are considered as molecular markers for CA-MRSA detection. SCC*mec* elements are important and serve as vehicle to transfer different genetic markers and genes that mediate virulence and resistance to antibiotics. The small mobile SCC*mec* IV or V genetic elements contain *mecA* gene and are transferred to other *S. aureus* strains more easily than the larger elements SCC*mec* I, II and III (Zhang et al.

2005). Our results revealed that 75% of MRSA harbored SCC*mec* IV, which may serve as an indication that those infections in children were mainly due to CA-MRSA. Alternatively types I, II and III known to be associated with HA-MRSA, accounted for 20% of the isolates undertaken in this study and were all PVL negative. Further classification of type IV into subtypes (a, b, c, d, e, g, and f) may play an essential role in understanding the epidemiology and thus help in the prevention and control of CA-MRSA clonal outbreaks (Zhang et al. 2005). SCC*mec* typing of the MRSA isolates in this study revealed predominance (53%) of subtype IV (a, b, c, d, g, h) followed by subtype IVe (22%), and subtype I (15%). Similarly Tokajian et al. (2010) revealed that SCC*mec* type IV was the major type among MRSA isolates from Lebanon. 86% of all nasal MRSA isolates from children were found to carry SCC*mec* IV. High prevalence of SCC*mec* IV (96%) was also reported by Lo et al. (2010) within PVL negative MRSA isolates. The results of this study additionally revealed that 35% of PVL negative MRSA recovered from nasal specimens harbored SCC*mec* IV, while it was 47% for the PVL positive ones. All the PVL positive isolates were MRSA harboring SCC*mec* IV except for one MSSA isolate, which agreed with the results obtained from Lebanon (Tokajian et al. 2010).

Deurenberg and Stobberingh (2008) showed that all *spa* t044 belonged to SCC*mec* IV. The combination of MLST with SCC*mec* typing was used as a tool to define clonal types of MRSA (Conceiçáo et al. 2009). Clustering *mec* typing and MLST revealed that the major MRSA clone in our samples was ST80-MRSA-IV with allelic variant 1-3-1-14-11-51-10. This clone is widely disseminated in Europe, Asia, and the Middle East and causes predominantly skin and soft tissue infections (Gillet et al. 2002). Larsen et al. (2008) found that the European CA-MRSA clone CC80-MRSA-IV primarily caused skin

and soft tissue infections in young people outside hospitals. ST30-MRSA-IVc is another MRSA clone detected in this study, which was also detected in Australia, Europe South America (Robinson and Enright 2004), and Lebanon (Tokajian et al. 2010). Vandenesch et al. (2003) reported that all PVL positive CA-MRSA isolates harbor SCC*mec* IV and clone ST80-MRSA-IV was detected among isolates recovered from France and Switzerland and other European countries (Wannet et al. 2005). Urth et al. (2005) on the other hand, reported the first isolation of clone ST80-MRSA-IV from a child in the Danish community. The clone was introduced on more than one occasion to families migrating from Middle East, and the infected child visited the Middle East with his mother few months before the appearance of the cluster for the first time in 1997. This was in harmony with the results of Larsen et al. (2008), who showed the high incidence of clone ST80-MRSA-IV in patients with recent history of travel or family relation to the Mediterranean or Middle East. Similarly, Rossney et al. (2007) reported that ST80 in Ireland was recovered from a patient from the Middle East. The wide dissemination of ST80 clone in Europe, Asia and other countries indicates that it is rapidly spreading all over the world.

In our study, the major MSSA types recovered were ST15 and ST22. These types however also included MRSA isolates, which could be and an indication of MSSA acquisition of some SCC*mec* type. Similarly, Robinson et al. (2005) has shown that ST36-MRSA-II has emerged from ST30-MSSA after the acquisition of SCC*mec* type II.

In conclusion, this study showed great diversity between MRSA and MSSA isolates and hence efficient epidemiological typing methods are essential to monitor and limit the occurrence and spread of epidemic clones between hospitals. Typing methods used should facilitate the discrimination between

unrelated isolates and the recognition of those belonging to the same lineages. Sequence-based *spa* typing can be used as a rapid screening test for MRSA. MLST and PFGE are the most reliable methods with *S. aureus*. MLST combined with SCC*mec* typing is essential to define clonal types of MRSA. Purpose, advantages and disadvantages differ between the four methods, but due to the progress in large scale sequencing methodologies, ease of data transfer and excellent comparability of results, DNA sequence-based typing techniques are still the ones being most frequently used, with the proper usage of appropriate methods facilitating effective epidemiological surveys.

This study has opened a research horizon that will enable researchers in Middle East to investigate the link between MRSA acquisition and various factors like age, hospitalization, antibiotic usage, and distinction between community- and hospital-acquired MRSA especially in infants. In order to limit the spread of *S. aureus*, systematic surveillance are required for isolates associated with hospital- and community-acquired infections. Due to limited studies dealing with the molecular typing of *S. aureus* in the region, larger numbers of isolates should be collected from different countries in order to better understand the pathogenesis, the combination of genes enhancing virulence and the spread and epidemiology of MSSA and MRSA. We hope that future studies will further elucidate the prevalence of MRSA carriage in children and the riskfactors associated with such carriage, will mark out its impact on transmission risk to patients, and will determine the risk of infection among children in the region .Additional studies in high-risk community populations are needed to identify the epidemiology of MRSA and to guide empirical treatment of suspected *S. aureus* infections in these populations.

Chapter 6

CONCLUSION

- This study was the first to employ *spa*, SCC*mec*, PFGE and MLST typing for the epidemiological characterization of MRSA and MSSA in Jordan.
- It reflects a high diversity in the genetic profile of MSSA (33 *spa* types compared to MRSA (17 *spa* types).
- MRSA infections in children have increased and several factors have shown to make children as hosts more susceptible to MRSA than adults.
- 103 isolates (15% PVL positive) were distributed over 63 nose and 40 stool specimens.
- *spa* typing revealed 48 different *spa* types with the most common being t044, t065, t386, and t223.
- BURP Analysis clustered the resulting 48 *spa* types into 8 different *spa* CCs and 8 singletons.
- 13 distinct allelic profile or STs were identified by MLST with the majority (17%) of the isolates belonging to ST80 followed by ST30, ST22 and ST15.
- SCC*mec* IV (a, b, c, d, g and h) predominated (53%) among MRSA isolates followed by subtype IVe (22%), and subtype I (15%).
- The major MRSA clone was ST80-MRSA-IV with allelic variant 1-3-1-14-11-51-10.

- 75% of MRSA harbored SCC*mec* IV, indicating that infection in Jordanian children were mainly due to CA-MRSA.
- Efficient epidemiological typing methods are essential to monitor and limit the occurrence and spread of epidemic clones between hospitals.
- Further larger parallel genetic studies with local MSSA and MRSA populations are necessary to understand the interactions of both populations.

Chapter 7

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Annex

List of all the *spa* types found among our isolates, along with their respective *spa* type %, clonal complexes (CCs) and ST types.

Serial number	Sample number	PVL	Methicillin	*spa*-type ID	Specimen	*spa*-type %	*spa*-CC	ST type/MLST
1	JS 10	neg-	susceptible	t002	nose	0.97	spa-CC 214	ST5
2	JS 22	neg-	susceptible	t005	nose	0.97	spa-CC 223	ST22
3	JS 19	neg-	susceptible	t008	nose	1.94	spa-CC 024/701	
4	JS 20	neg-	susceptible	t008	nose		spa-CC 024/701	ST30
5	JS 52	neg-	susceptible	t012	nose	1.94	spa-CC 012	ST30
6	JS 53	neg-	susceptible	t012	stool		spa-CC 012	
7	JS 04	neg-	resistant	t018	nose	1.94	spa-CC 012	
8	JS 05	neg-	resistant	t018	stool		spa-CC 012	
9	JS 01	neg-	susceptible	t021	nose	1.94	spa-CC 012	ST30
10	JS 70	neg-	resistant	t021	nose		spa-CC 012	ST30
11	JS 56	neg-	susceptible	t024	stool	0.97	spa-CC 024/701	ST8
22	JS 105	Pos+	resistant	t044	stool		spa-CC 044	
12	JS 42	Pos+	resistant	t044	stool	10.67	spa-CC 044	
13	JS 45	Pos+	resistant	t044	nose		spa-CC 044	ST80
14	JS 46	Pos+	resistant	t044	stool		spa-CC 044	
15	JS 60	Pos+	resistant	t044	stool		spa-CC 044	ST80
16	JS 69	Pos+	resistant	t044	nose		spa-CC 044	
17	JS 78	Pos+	resistant	t044	nose		spa-CC 044	
18	JS 83	Pos+	resistant	t044	stool		spa-CC 044	
19	JS 95	Pos+	resistant	t044	stool		spa-CC 044	

20	JS104	Pos+	resistant	t044	nose		spa-CC 044	
21	JS106	Pos+	resistant	t044	nose		spa-CC 044	
23	JS 03	neg-	susceptible	t065	nose	9.7	spa-CC 065	ST46
24	JS 06	neg-	susceptible	t065	nose		spa-CC 065	
25	JS 26	neg-	susceptible	t065	stool		spa-CC 065	
26	JS 58	neg-	susceptible	t065	stool		spa-CC 065	
27	JS 66	neg-	susceptible	t065	nose		spa-CC 065	
28	JS 73	neg-	susceptible	t065	nose		spa-CC 065	
29	JS 74	neg-	susceptible	t065	stool		spa-CC 065	
30	JS 90	neg-	susceptible	t065	nose		spa-CC 065	
31	JS 91	neg-	susceptible	t065	stool		spa-CC 065	
32	JS107	neg-	susceptible	t065	stool		spa-CC 065	
33	JS 38	neg-	susceptible	t084	nose	2.91	spa-CC 346/2398/084	
34	JS 54	neg-	susceptible	t084	nose		spa-CC 346/2398/084	ST15
35	JS 75	neg-	susceptible	t084	nose		spa-CC 346/2398/084	
36	JS 71	Pos+	resistant	t131	nose	0.97	spa-CC 044	ST80
37	JS 43	neg-	susceptible	t1414	nose	0.97	spa-CC 012	
38	JS 81	neg-	susceptible	t1614	nose	0.97	spa-CC 1614	
39	JS 50	neg-	resistant	t1651	nose	1.94	no founder	ST395
40	JS 51	neg-	resistant	t1651	stool		no founder	
41	JS 65	neg-	susceptible	t166	nose	0.97	Singleton	ST97
42	JS 44	neg-	resistant	t214	nose	2.91	spa-CC 214	
43	JS 77	neg-	resistant	t214	nose		spa-CC 214	ST5
44	JS 87	neg-	resistant	t214	nose		spa-CC 214	
45	JS 15	neg-	resistant	t223	nose	6.79	spa-CC 223	ST22
46	JS 28	neg-	susceptible	t223	stool		spa-CC 223	

47	JS 55	neg-	resistant	t223	stool		spa-CC 223	ST22
48	JS 72	neg-	susceptible	t223	nose		spa-CC 223	
49	JS 86	neg-	susceptible	t223	stool		spa-CC 223	
50	JS 94	neg-	susceptible	t223	nose		spa-CC 223	
51	JS 99	neg-	resistant	t223	stool		spa-CC 223	
52	JS 79	neg-	susceptible	t2313	stool	0.97	spa-CC 1614	
53	JS 41	neg-	susceptible	t2398	nose	0.97	spa-CC 346/2398/084	
54	JS 61	neg-	susceptible	t267	nose	2.91	Singletons	ST1
55	JS 62	neg-	susceptible	t267	stool		Singletons	
56	JS 63	neg-	susceptible	t267	stool		Singletons	
57	JS 21	neg-	susceptible	t3096	stool	0.97	spa-CC 1614	
61	JS 33	neg-	susceptible	t330	nose		spa-CC 065	
58	JS 64	neg-	susceptible	t330	nose	3.88	spa-CC 065	ST46
59	JS101	neg-	susceptible	t330	nose		spa-CC 065	
60	JS102	neg-	susceptible	t330	stool		spa-CC 065	
65	JS 100	neg-	susceptible	t338			spa-CC 012	
62	JS 37	neg-	susceptible	t338	nose	3.88	spa-CC 012	
63	JS 48	neg-	susceptible	t338	nose		spa-CC 012	
64	JS 49	neg-	susceptible	t338	stool		spa-CC 012	
66	JS 29	neg-	susceptible	t346	nose	2.91	spa-CC 346/2398/084	
67	JS 31	neg-	susceptible	t346	nose		spa-CC 346/2398/084	ST15
68	JS 32	neg-	susceptible	t346	stool		spa-CC 346/2398/084	ST15
69	JS 07	neg-	resistant	t386	stool	7.76	Excluded	ST80
70	JS 11	neg-	resistant	t386	nose		Excluded	
71	JS 12	neg-	resistant	t386	nose		Excluded	

72	JS 13	neg-	resistant	t386	stool		Excluded	
73	JS 23	neg-	resistant	t386	stool		Excluded	
74	JS 39	neg-	resistant	t386	nose		Excluded	
75	JS 92	neg-	resistant	t386	nose		Excluded	
76	JS 93	neg-	resistant	t386	stool		Excluded	
77	JS103	Pos+	resistant	t416	nose	0.97	Excluded	
78	JS 02	neg-	resistant	t5700	nose	0.97	spa-CC 223	
79	JS 40	neg-	susceptible	t5702	nose	0.97	spa-CC 065	
80	JS 76	neg-	susceptible	t5703	stool	0.97	Singletons	
81	JS 59	neg-	susceptible	t5704	nose	0.97	spa-CC 223	
82	JS 18	neg-	resistant	t5839	stool	0.97	spa-CC 214	ST5
83	JS 24	neg-	resistant	t5840	stool	0.97	spa-CC 024/701	
84	JS 89	neg-	susceptible	t5841	stool	0.97	spa-CC 044	
85	JS 85	neg-	susceptible	t5842	stool	0.97	no founder	ST34
86	JS 30	neg-	susceptible	t5843	stool	0.97	spa-CC 346/2398/084	
87	JS 98	neg-	resistant	t5844	nose	0.97	spa-CC 223	
88	JS 25	Pos+	susceptible	t5845	stool	0.97	Excluded	ST395
89	JS 57	neg-	susceptible	t5846	nose	0.97	spa-CC 012	
90	JS 96	neg-	susceptible	t5847	stool	0.97	spa-CC 223	
91	JS 80	neg-	susceptible	t5848	nose	0.97	spa-CC 065	
92	JS 82	Pos+	resistant	t5849	nose	0.97	spa-CC 044	ST80
93	JS 97	neg-	susceptible	t5884	nose	0.97	spa-CC 223	
94	JS 84	neg-	resistant	t6081	nose	0.97		ST1
95	JS 34	neg-	susceptible	t6082	nose	0.97	spa-CC 346/2398/084	
96	JS 16	neg-	susceptible	t701	nose	3.88	spa-CC 024/701	

97	JS 17	neg-	susceptible	t701	stool		spa-CC 024/701	ST6
98	JS 35	neg-	susceptible	t701	stool		spa-CC 024/701	
99	JS 36	neg-	susceptible	t701	nose		spa-CC 024/701	
100	JS 27	neg-	susceptible	t712	nose	0.97	spa-CC 223	
101	JS 47	neg-	resistant	t790	nose	0.97	spa-CC 223	ST22
102	JS 08	neg-	resistant	t922	nose	1.94	Singletons	ST25
103	JS 09	neg-	resistant	t922	nose		Singletons	