Molecular Typing of *Staphylococcus aureus* and Investigation of Biofilm Formation of Isolates Collected from Jordan

by

Dana A. Bazzoun

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science
Molecular Biology
Lebanese American University
2009-10

Under the supervision of
Dr. Sima Tokajian

LEBANESE AMERICAN UNIVERSITY

Thesis approval Form (Annex III)

Student Name: Dana Bazzoun I.D. #: 200803738

Thesis Title : Molecular Typing of *Staphylococcus aureus* and Investigation of Biofilm Formation of Isolates Collected from Jordan

Program : M.Sc Molecular Biology

Division/Dept : Natural Sciences/Biology

School : **School of Arts and Sciences**

Approved by: [redacted]

Thesis Advisor: Dr. Sima Tokajian

Member : Prof. Fuad Hashwa

Member : Dr. Brigitte Wex

Date 27/9/2010

I grant to the **LEBANESE AMERICAN UNIVERSITY** the right to use this work, irrespective of any copyright, for the University's own purpose without cost to the University or to its students, agents and employees. I further agree that the University may reproduce and provide single copies of the work, in any format other than in or from microfilms, to the public for the cost of reproduction.

Dana A. Bazzoun

LEBANESE AMERICAN UNIVERSITY

ABSTRACT

Molecular Typing of *Staphylococcus aureus* and Investigation of Biofilm Formation of Isolates Collected from Jordan

by Dana A. Bazzoun

Staphylococcus aureus has long been recognized as an important pathogen associated with different types of diseases in humans. The increase in the frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) as the causal agent of nosocomial infection and the possibility of emergence of resistance to vancomycin demands a quick and trustworthy characterization of isolates and identification of clonal spread within hospitals. In this study 68% of clinical *S. aureus* isolates (n=60) collected from Jordan were MRSA. Molecular typing of those isolates was done through *spa* typing, pulsed field gel electrophoresis (PFGE). It was found that each of these typing techniques had a certain discriminatory power and thus a combination of both would be essential for proper epidemiological studies aiming at understanding and controlling the spread of infections specially in the hospital environment. *spa* typing showed that *spa* type t044 was the most common type and represented 28% of the isolates studied and 38% of the MRSA population. PFGE revealed that isolates having the same *spa* type do not necessarily have the same banding patterns. PFGE has shown to have a higher discriminatory power than *spa* typing; different PFGE banding patterns were detected for samples of the different and/or the same *spa* types.

Evidences that *S. aureus* has the ability to form matrix-encased biofilms, which further contributes to its pathogenicity through different mechanisms, are increasing, thus it was inevitable to look for potential biofilm formers among those isolates. Further studies however, elucidating the mechanism of biofilm formation are merited to reduce nosocomial as well as other types of infections. In conclusion, sequence-based *spa* typing shows comparative sensitivities to PFGE, and is a rapid and easy handling method. The sequence-based *spa* typing can be used as a rapid screening test when MRSA outbreak is suspected in areas and hospitals. On the other hand, *spa*, Multi locus sequence typing (MLST) sequences, and PFGE pattern database developed in this study will serve as a basis for information for the long term evolutionary and epidemiological studies of local *S. aureus* recovered not only in Jordan, but also in neighboring countries including Lebanon. Systematic surveillance of both hospital and community isolates in Jordan and Lebanon together with measures designed to limit the spread are required.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table Page

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*

MSCRAMM: microbial surface components recognizing adhesive matrix molecules

MLST: Multi-locus sequence typing

MRSA: methicillin-resistant *Staphylococcus aureus*

MSA: Mannitol Salt Agar

MSSA: methicillin-sensitive *Staphylococcus aureus*

OD: optical density

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

ACKNOWLEDGEMENTS

I would like to thank all the people who made these two years at LAU possible and memorable.

First of all, I would like to convey my greatest and genuine appreciation to Dr. Sima Tokajian. I am very indebted for your unlimited support, help, encouragement and friendship. You were always around when I needed you, you shared my success and my failure my happiness and my sadness. Thanks million times for all what you did to me. None of this would have been possible if it wasn't for your endless motivation. I am also grateful to the Dean of Arts and Science, Prof. Fuad Hashwa, faculty members of the Biology Department: Dr. Constantine Daher, chairperson of the department, Dr. Brigitte Wex Dr. Mirvat EL-Sibai, , Dr. Pierre Zalloua, Dr. Ralph Abi-Habib, Miss Helena Abou Farah for their positive role they have played during my Master's program and a special thanks for a special professor and friend Dr. Roy Khalaf. I am deeply thankful to Miss Maya Farah, for her advice, help, encouragement, and sincere friendship. We shared a lot of experiences that I will never forget. I would also like to thank my friends and class mates for making it such an enjoyable experience.

Last but not least, I would like to show my deep and sincere love and appreciation to my family. I thank them for supporting and encouraging me every single day, believing in me and the decisions I took, and pushing me to becoming the most I can be, Sara and Ghassan for being the best sister and brother ever, mom and dad for dedicating themselves so that we become the best of ourselves.

I dedicate this work to my father.

Chapter 1

INTRODUCTION

S. aureus has been identified in the 1880s where it was associated with a wide range of serious and mild clinical manifestations (Deurenberg and Stobberingh 2008). *S. aureus* specifically is a major cause of many serious nosocomial and CA (community acquired) human and animal infections, where it colonizes mainly the nasal passages and regularly other anatomical locations, including the skin, oral cavity and gastrointestinal tract (Todar 2008). Bacteremia, necrotizing pneumonia, endocarditis, and deep abscess formation and even death may result from an infection (Schouls et al. 2009). The constant evolution of antimicrobial resistance such as methicillin-resistant strains of *S. aureus* (MRSA) has caused serious invasive and life-threatening infections in young children (Boyle-Vavra and Daum 2007). Pathogenicity of *S. aureus* is correlated with several virulence factors such as Protein A, staphylococcal enterotoxins, exfoliative toxins, toxic shock toxin syndrome toxin 1 (TSST-1), Pantone Valentine Leukocidin (PVL), leukotoxin (LukE LukD), adhesion factors that are involved in initiating series of immune reactions in the host. In addition to all of these, *S. aureus'* vital infectious component is attributed mainly to its ability to form biofilms which is induced by an adhesion of bacteria to a surface, followed by accumulation in multilayered cell clusters through intercellular adherence (Izano at al. 2008).

The increase in the frequency of MRSA as the causal agent of nosocomial infection and the possibility of emergence of resistance to vancomycin demands a quick and trustworthy characterization of isolates and

identification of clonal spread within hospitals (Vindel et al. 2009). Thus proper epidemiological studies, using molecular approaches, are vital to understand *S. aureus* distribution and relatedness to design rational pathogen control methods. There have been several studies conducted for this purpose in Tehran and Turkey and only one in the Arab world, Lebanon, to characterize *S. aureus* in the region (Tokajian et al. 2010) where different molecular approaches were employed including *spa* typing, MLST, and Mec Subtyping. Each of these techniques had its own sensitivity, specificity and precision.

This study represents the first report in Jordan and the Arab world that compares Pulse Field-Gel Electrophoresis and *spa* typing as two major molecular typing techniques used for *S. aureus* characterization. It also screens for potential biofilm formers among isolates to better understand the genetic mechanism that lies behind this potential.

OBJECTIVES

- Investigate the clonal structure and molecular characteristics of MSSA and MRSA isolated from children in Jordan.
- Assess the molecular Epidemiology of *S. aureus* isolates associated with infections in children.
- Determine the frequency of PVL-producing *S. aureus* in Jordan.
- *spa* typing in combination with Based Upon Repeat Pattern (BURP) grouping analysis for epidemiological typing of *S. aureus*.

Chapter 2

LITERATURE REVIEW

2.1 Overview of *Staphylococcus aureus*

Staphylococci are gram-positive, facultative anaerobes, and spherical bacteria that occur in microscopic clusters resembling grapes. *Staphylococcus aureus* is a gram positive staphylococcal species that has been linked to serious nosocomial and community acquired human as well as animal infections. Strains belonging to *S. aureus* are distinguished by growth properties, specific surface factors, and the ability to form a clot in the presence of fibrinogen due to the production of coagulase enzyme. It colonizes mainly the nasal passage and regularly in other anatomical locations, including the skin, oral cavity and gastrointestinal tract (Todar 2008). *S. aureus* has been recognized in the 1880s and associated with a wide range of serious and mild clinical manifestations (Deurenberg and Stobberingh 2008). It is one of the most common pathogens in the United States, where 3 out of 10 individuals have their mucous membranes colonized with *S. aureus* (Jiyeon 2009). Since humans are major reservoirs of *S. aureus*, the individual can be a long period or a discontinuous carrier of the pathogen (Jiyeon 2009). More often those carriers do not show any of the clinical symptoms; however bacteremia, necrotizing pneumonia, endocarditis, and deep abscess formation and even death may result from an infection (Schouls et al. 2009).

2.2 Occurrence

Morbidity rate due to *S. aureus* infection was around 80% in the 1940s, before any use of antibiotic such as methicillin (Deurenberg and Stobberingh 2008). Today *S. aureus* remains a leading cause of health care associated infections, as well as community-acquired infections (Tenover et al. 2005). Like any living organism, the stress exerted on *S. aureus* with the introduction of multiple drugs and the extensive use of antibiotics in health care facilities against it made it inevitable to develop resistance against such drugs and thus the rise of methicillin resistant strain. According to Levy and Marshall (2004), more than 95% of all *S. aureus* isolates possess resistance to penicillin, and 40–60% of clinical isolates in the United States of America and the United Kingdom exhibit methicillin resistance. Studies on US populations showed that by 2005 the rate of invasive MRSA infections reached 31.8 per 100,000 individuals (Klevens et al. 2007). The extensive spread of MRSA in the community and the threat of increasing cases of severe infection is a matter of concern and requires search for new control measures (Vindel at al. 2009). Other environmental factors that might increase the stretch of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) are contaminated fomites, such as shared razors, towels, soap bars, whirlpools used by athletes. Additionally stethoscopes, pagers, bed spaces, workstations contamination are involved in the spread of health care associated methicillin-resistant *S. aureus* (HA-MRSA) (Decker 2008).

2.3 Pathogenesis of *Staphylococcus aureus*

Pathogenicity of *S. aureus* is correlated with several virulence factors such as proteins, toxins, and adhesion factors that are involved in initiating

series of immune reactions in the host. Some of the virulence factors recognized are thermonuclease, hemolysins (α, ß, δ, and γ), lipase, hyaluronidase which act mainly to invade host tissues (Sandel and McKillip 2004; Kuroda et al. 2007). In addition to enzymes, there is a large group of toxins including staphylococcal enterotoxins, exfoliative toxins, toxic shock toxin syndrome toxin 1 (TSST-1), Pantone Valentine Leukocidin (PVL), leukotoxin (LukE, LukD), epidermolysins A and B and others (Baba-Moussa et al. 2008).

PVL and other leukocidins, belong to the family of "bicomponent synergohymenotropic toxins" which are two-component pore-forming toxins that eventually cause 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). PVL elevates the release of inflammatory mediators such as leukotriene B4, IL-8 and histamine from monocytes and macrophages, their major cell target (Lina et al. 1999). Three exfoliative toxins (ETA, B and D) cause exfoliation of the epidermis by cleaving a desmosomal protein predominant in granular layer, desmoglein 1, without any signs of skin inflammation (Ladhani et al. 1999).

Some *S. aureus* isolates produce TSST-1, a superantigen toxin, encoded by *tst* gene. It is the major cause of toxic shock syndrome, staphylococcal scarlet fever, and neonatal toxic shock-like exanthematous diseases (Kikuchi et al. 2003). This superantigen has the ability to cross-link the class II major histocompatibility complex of antigen-presenting cells and the T-cell receptor (TCR) b-chain variable regions. This in turn leads to the nonspecific activation of 20% to 25% of the T-cell as opposed to 0.1% specifically activated T-cells by a conventional allergen. IL-4, IL-5, IL-13, and

many others, are thus produced leading to a local IgE production and inflammation (Patou et al. 2008).

Clearly those locally produced toxins and virulence factors add up to the virulence and pathogenesis of *S. aureus*, yet not all pathogenic isolates of *S. aureus* contain the genes coding for these toxins. On the other hand, a common virulence factor encoded by all *S. aureus* isolates' genomes was designated as protein A. Protein A is a conserved surface protein present in all *S. aureus* strains (Martin et al. 2009). Protein A is a 42-kDa molecule that could be either secreted or membrane-associated and interrelates with a range of human and animal immunoglobulins (Silverman and Goodyear 2006). Protein A binds to the Fc-region of IgG to prevent phagocytosis, and according to several studies it provokes enormous inflammatory responses in human airway and corneal epithelial cells (Gomez et al. 2004) through eliciting T cell-independent B cell proliferation (Bekeredjian-Ding et al., 2007).

2.4 *Staphylococcus aureus* biofilm

S. aureus' vital infectious component is attributed mainly to its ability to form a biofilm (Izano at al. 2008). Biofilms enhances the bacterial survival under demanding conditions such as low nutrient availability, antibiotic and oxidative stress, and host immune defenses (Mackey-Lawrence et al. 2009). Biofilm formation occurs in several stages. It requires adhesion of bacteria to a surface, followed by accumulation in multilayered cell clusters through intercellular adherence (Corrigan et al. 2007). The first step is mediated through adhesins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Tormo at al. 2007). MSCRAMMs

function through attachment to different cell types and abiotic surfaces (Cucarella et al. 2004). MSCRAMMs include clumping factors A and B (ClfA and ClfB), protein A and the fibronectin binding proteins A and B (FnBPA and FnBPB) that are characterized by an N and C terminal signal sequences for secretion and cell wall-anchoring by sortase respectively (Corrigan et al. 2007). The *icaABCD* cluster, an operon present in *S. epidermidis* and *S. aureus*, encodes proteins that assist in the intercellular adhesion step of biofilm formation entailed in the synthesis of the biofilm matrix polysaccharide, poly-*N*-succinyl b-1-6 glucosamine (PIA-PNSG) (O'Neill et al. 2008). The *(ica)* locus and PIA/PNAG biosynthesis regulation mechanism is yet not finalized and potential research in this perspective is still of particular interest (O'Gara 2007). Additional surfactant proteins are being recognized, beyond the *ica* locus, as key factors involved in generating staphylococcal biofilm (Otto 2008) such as *agr, arl, svr, srr, sae, mgrA, sarA, sarA-homologs, rot, sarR, sarS, sarT, sarU, sarV, and sarX* (Fournier and Hooper 2000; Luong and Lee 2003; Manna and Cheung 2001; Manna et al. 2004; McNamara et al. 2000; Recsei et al. 1987; Schmidt et al. 2001; Tegmark et al. 2000; Yarwood et al. 2001). These recent advances necessitate the introduction of anti-staphylococcal drugs and vaccines to eradicate resistant biofilms.

2.5 Resistance to antibiotics

Resistance to methicillin is primarily due to ß-lactamase enzyme, which hydrolytically cleaves the ß-lactam ring present in penicillin. Normally, *S. aureus* produce four types of membrane-bound transpeptidase proteins known as penicillin-binding proteins (PBPs 1–4) that control the final stages of cell wall biosynthesis. Thus the main role of the ß -lactam antibiotics is to disrupt the activity of the PBPs (Murphy et al. 2008). However, MRSA contain a gene called *mecA* that codes a 78-kDa penicillin-binding protein

(PBP) 2a (or PBP2') that binds penicillin and inhibits its function to prevent the peptidoglycan synthesis disruption. The *mecA* gene, 2.1 Kb gene, is integrated in a mobile genetic element referred to as staphylococcal cassette chromosome *mec* (SCC*mec*) (Deurenberg and Stobberingh 2008). SCC*mec* contains the *mec* gene complex that itself includes the *mecA* gene along with regulatory elements *mecI* (encodes MecI, repressor protein) and *mecR1* (encodes MecR1, signal transducer protein), the *ccr* gene complex which codes for a recombinase, *ccrA, ccrB, or ccrC,* in charge of the mobility of the whole cassette. The rest of the cassette includes nonessential components called *J* regions (*J1, J2,* and *J3*) that separate the *mec* and *ccr* gene complexes and display antibiotic resistance properties (Zhang et al. 2009). Thus the overall structure of the SCC*mec* is J1-*ccr*-J2-*mec*-*J3* (Milheirico et al. 2007). The cassette is located within a specific integration sequence (ISS) of *orfX* that has an unknown function upstream of *spa* gene (Berglund et al. 2008).

2.6 Molecular typing of *Staphylococcus aureus*

Due to the massive dissemination of MRSA in hospital facilities and the community, taking protective measures is a major concern. For scientists to understand the pathogen distribution and route of the spread, it is inevitable as a primary step to trace the origin and type of the bacterial strain to identify any epidemiological and genetic relatedness. Thus typing is necessary to identify lineages and profiles of MRSA as well as to discriminate between a HA-MRSA and CA-MRSA. In general, we have the "band-based" and "sequence- based" techniques, with the latter allowing for data exchange between countries (Deurenberg and Stobberingh 2008). The most common techniques used for typing *S. aureus* include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *spa* typing, and SCC*mec*

subtyping (Aires de Sousa and De Lencastre 2004). The purpose of each differs, with all having advantages and disadvantages.

2.6.1 Pulsed-field gel electrophoresis

It is one of the common typing techniques, used first by Schwarz and Cantor in 1984, and is based on the digestion of the bacterial chromosomal DNA with a restriction enzyme that recognizes specific sites, generating large fragments of DNA (10-800 Kb) to be separated by agarose gel electrophoresis in an alternating voltage gradient electric field (Trindade et al. 2003). PFGE of *S. aureus*, the analysis of the banding pattern of the *SmaI*-digested DNA by specific software will produce different migration profiles used to identify the strain of this organism. PFGE is important in revealing population structures and classifying *S. aureus* outbreaks (Narukawa et al. 2009). Yet *S. aureus* strains are impossible to tell apart using PFGE when investigating local outbreaks (Patel et al. 2008). According to a study carried by Merlin et al. (2009), PFGE was inferior to *spa* typing with respect to speed, reproducibility, accessibility, ease of use, and standardized nomenclature. The fact that PFGE results are inconsistence due to unharmonized protocols among laboratories, PCR based techniques suit interlaboratory comparisons more than PFGE (Schouls et al. 2009).

2.6.2 Multi-locus sequence typing

To track the evolutionary change of a pathogen, scientists scan housekeeping genes that are considered to be conserved loci along the genome. These loci are known to accumulate changes slowly through time; this will easily reflect the evolutionary pattern of the pathogen but will

decrease the ability to differentiate between closely related species (Cooper and Feil 2004). Such loci are good targets for multi-locus sequence typing (MLST) being proposed in 1998 as a tool to supply accurate, portable data about the evolutionary history and biology of bacterial pathogens (Urwin and Maiden 2003). The study of *S. aureus*'s evolutionary history using MLST select for 500-bp fragments of seven housekeeping genes which are *arc, aroE, glpF, gmk, pta, tpi, and yqiL* (Deurenberg and Stobberingh 2008). After sequencing the gene fragments, the data is analyzed and strains are classified into clonal complexes CC, with the ancestral CC being the strain with the highest number of single locus variants (SLVs) (Cooper and Feil 2004). Different strains are combined into the same clonal complex when five of the seven genes are identical (Deurenberg and Stobberingh 2008). MLST remains to be an expensive and laborious technique that needs to be updated regularly, but it is very efficient in phylogenetic studies with the data generated being interchangeable among different laboratories (Te Witt et al. 2009).

2.6.3 Typing of spa locus

Another molecular technique based on DNA sequencing is Staphylococcal Protein A *(spa)* typing. It involves sequencing a single short repeat region in the gene locus region that encodes Protein A (Khandavilli et al. 2008). This region is characterized by the presence of repeats that vary in number and sequence due to point mutations, deletions, and duplications of the repeats (Kahl et al. 2005). The sequence of the repeats will create a profile used for clustering *S. aureus* isolates and consequently establishing a large internet database that could be accessible by various laboratories (Schouls et al. 2009). This provides epidemiological information for local investigations of *S. aureus* (Hallin et al. 2007). Since *spa* typing targets only one locus of the

whole genome, it is considered more advanced than PFGE in terms of speed, ease of interpretation and comparison between laboratories (Ruppitsch et al. 2006), but inferior to it in terms of discriminatory power (Kuhn et al. 2007). On the other hand, and according to Heyn et al. (2002), it is considered more discriminatory than MLST.

2.6.4 SCCmec typing

Since methicillin resistance is an acquired mechanism among *S. aureus* strains, identifying the mobile genetic element responsible for this resistance is necessary in order to describe MRSA lineages along their genetic background (Chmelnitsky et al. 2007). To date, there exists five classes of *mec* gene complexes designated as A, B, C1, C2, and D, in addition to five allotypes of the *ccr* complex chosen to be type 1,2,3,4, and 5 (Ito et al. 2003) which arrange in several combinations to form a different SCC*mec* type (Oliveira et al. 2006). Eight types of SCC*mec* are recognized till now (SCC*mec* I, II, III, IV, V, VI, VII, and recently VIII) (Zhang et al. 2009) ranging in size from 20.9 to 66.9 Kb (Deurenberg and Stobberingh 2008). Furthermore, differences in the *J* regions will sub-type the SCC*mec* types into variants (Ma et al. 2002). Of these 8 types, SCC*mec* II and III are correlated with hospital-acquired MRSA, while SCC*mec* IV and V are associated with community-acquired MRSA that produce PVL (Zetola et al. 2005).

Chapter 3

MATERIALS AND METHODS

3.1 Clinical isolates

A total of 60 *S. aureus* isolates were obtained from clinical specimens in the period between years 2009 and 2010. These isolates were kindly provided by Dr. Asem Shihabi from the University of Jordan hospital in Amman-Jordan. They have been isolated from different sites of infection including mainly blood, wounds, catheters, burns, and nasal and breast swabs. Patients are distributed along a wide age range (one day up to 43 years). Isolates included both MRSA and MSSA. The samples were streaked on Mannitol Salt Agar (MSA) to be further purified and stored in Cryobanks at -20°C and -80°C. The isolates were designated by JS D 1- JS D 60.

3.2 DNA Extraction

Isolates were grown overnight on MSA at 37°C. DNA extraction was carried using Nucleospin® Tissue and BIORAD InstaGene™ Matrix according to the manufacturer's instructions. The extracted DNA was stored at -20°C until needed.

3.3 16S rRNA, lukS-PV, mecA genes amplification

All polymerase chain reaction (PCR) assays were performed on a PerkinElmer 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'-

AACTCTGTTATTAGGGAAGAACA-3') and Staph750R (5'-CCACCTTCCTCCGGTTTGTCACC-3'). The *lukS-PV* genes were amplified using Luk-PV-1 (5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and Luk-PV2 (5'-GCATCAAGTGTATTGGATAGCAAAAGC-3') primers, and the *mecA* gene was amplified using MecA1 (5'-GTAGAAATGACTGAACGTCCGATAA-3') and MecA2 (5'-CCAATTCCACATTGTTTCGGTCTAA-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 set at 94°C for 5min followed by 10 cycles of 94°C for 45 s, 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 a 1.5% agarose gel using 1 X 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 5-10 µL 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 enzyme is 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 required 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 PFGE

All the 60 isolates 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 an attempt to optimize the protocol furthermore. Two to three colonies were inoculated in a 5 mL Brain Heart Infusion Broth overnight. Then 1mL of each cell suspension is centrifuged at 15, 000 x g for 1min and the pellet was resuspended in a 0.5mL of TEN buffer. After 1min centrifugation at 15, 000 x g for 1min, the pellet was then resuspended in 0.5mL of EC 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 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. The slice was then 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 cell 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 after staining with EtBr.

3.6 Biofilm assay for S. aureus

One colony of each sample was incubated in 5ml overnight culture of Brain Heart Infusion (BHI), in a shaking water bath 37°C. The broth was diluted 1:200 in BHI including 0.25% glucose (Izano et al. 2008 with minor modifications). 200µL was transferred to each well using a 96 well cell star plate (each sample has 4 wells). The plate was then incubated for 25hrs at 37°C. Medium was then discarded and washed twice with 1X PBS after beating the plate twice. 100µL of each of the enzyme solutions (DNase, proteinase K, and sodium metaperiodate) was added into each of the three wells leaving the fourth as a control and incubated for 3hrs at 37°C. Plate was then washed once with 1X PBS and stained for 15min with 1% crystal violet (200µL/well), followed by washing twice with 1X PBS and finally 200µL EtOH/acetone 80:20 was added. OD590nm (optical density) was read with ELISA reader. Controls: Positive: S. epi RP62A, negative:TM300, blank:BHI + 0.25% glucose. The positive and negative controls were kindly donated by Dr. Barbara Kahl form University of Munster, Germany.

Chapter 4

RESULTS

4.1 16S rRNA, lukS-PV, mecA genes amplification

To identify the isolates taken from Jordan a multiplex PCR for the 16S rRNA, *lukS-PV* and *mecA* was carried to differentiate MRSA from MSSA and indicate whether each isolate is a PVL producer or not. The gels showed strains with 3 bands, 2 bands or 1 band. The first band corresponds to a 756bp size and refers to the partial amplification of the 16S rRNA gene compatible with that obtained by McClure et al. (2006). The second band lies between the 400 and 500bp molecular marker bands, specifically it is a 433bp marker and is the amplification product of the *PVL* gene. The third band corresponds to the amplification product of the *mecA* gene and is a 310bp band. The results were also well-matched with those obtained by McDonald et al. (2006).

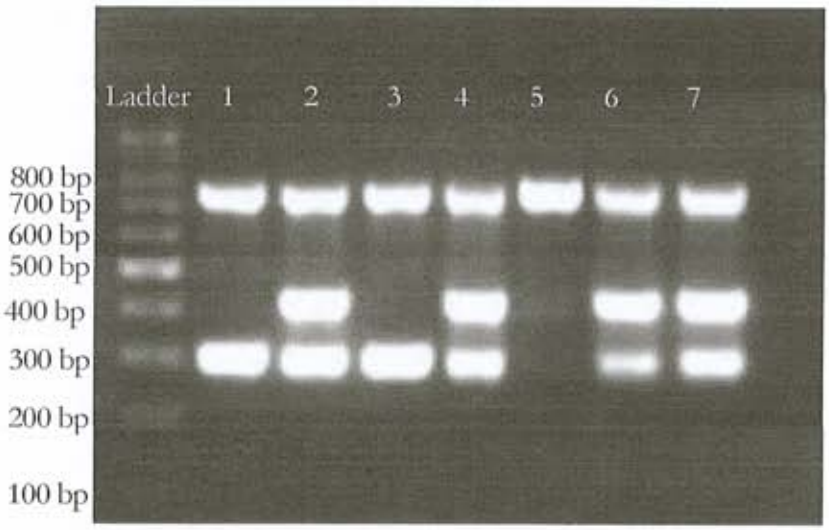


Fig 1. 1.5% agarose gel electrophoresis showing representative PCR products after 16S rRNA, *lukS-PV*, *mecA* genes amplification. The first lane shows the DNA ladder (100bp); lanes 1-7 show PCR products of the three genes of different isolates. Lane 8 is the negative control.

Table 1 represents the results of the triplex PCR for all the 60 isolates. It showed that 68% (41 samples) of the 60 isolates were MRSA and the remaining 32% (19 samples) were MSSA. 42 isolates were PVL negative (70%) and the remaining 30% (18 isolates) were PVL positive. It illustrates that of the 18 PVL positive isolates, 15 were MRSA making up to 83% of the PVL positive samples and the remaining 17% were MSSA (3 out of 18).

Table 1. The distribution of isolate among MRSA and MSSA, PVL positive and PVL negative.

	MRSA	MSSA
Number of samples	41 (68%)	19 (32%)
PVL	**Negative**	**Positive**
Number of samples	42 (70%)	18 (30%)
	MRSA	**MSSA**
Number of PVL positive	15 (83%)	3 (17%)

4.2 spa typing

The bands obtained for all 60 isolates, bands obtained after the *spa* gene amplification varied in size between 250 and 600bp depending on the number of repeats within the gene (Fig 2).

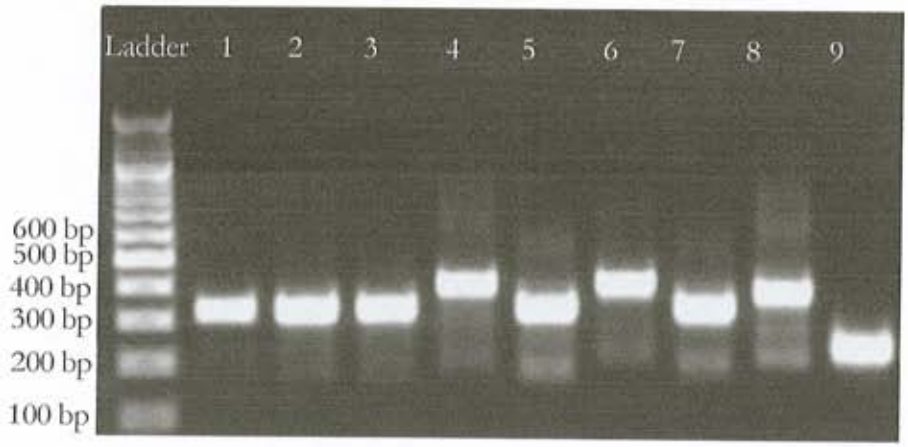


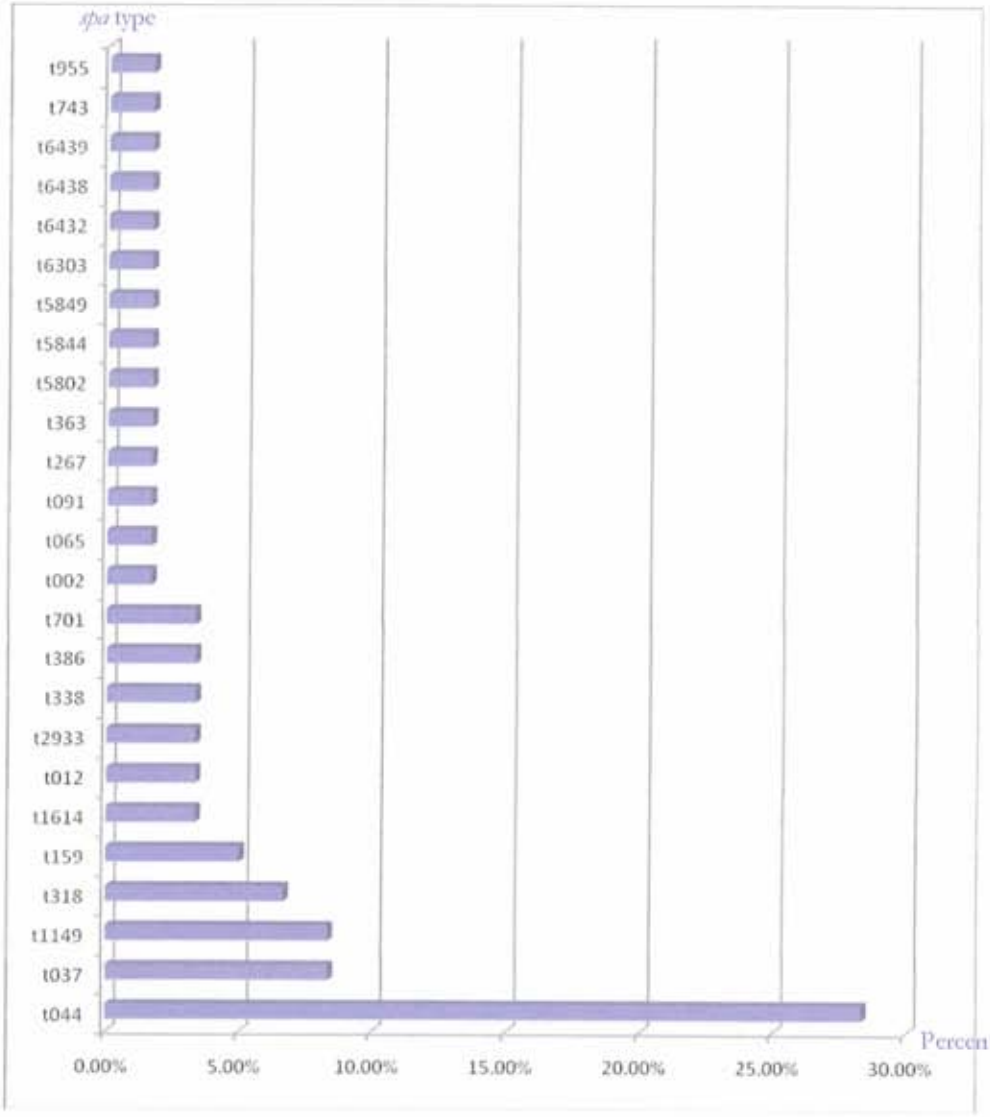
Figure 2. 1.5% gel electrophoresis showing PCR products after *spa* gene amplification. L:100 bp ladder; samples 1-9 amplified *spa* gene with sizes between 250 bp and 450 bp.

All 60 isolates were typeable. *Spa* sequence typing was essentially performed according to RIDOM protocol using RidomStaphType Version (1.5) software. The analysis revealed 25 different *spa* types with the most common being t044 representing 28% of the isolates. The other most common *spa* types included: t037, t1149, t318, t159, t1614, t012 representing 8%, 8%, 7%, 5%, 3%, and 3% of the studied isolates, respectively (Fig 3).

Within the MRSA 39% were of *spa* type t044 and 12.19% were *spa* type t037 as opposed to 5.26% and none within the MSSA (Fig 4).

Using BURP algorithm, the 25 *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 2 major *spa* CCs, 8 singletons, 2 clusters with no founder, and one *spa* type (t386) was excluded (the algorithm only clusters the *spa* types with 5 repeats and more) (Fig 5).

Fig 3. The 25 spa types with the corresponding number and percentage of samples of each.



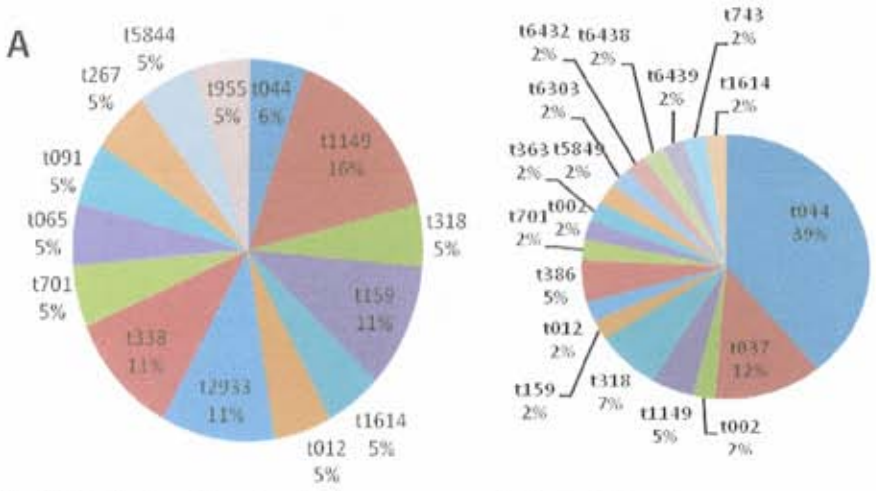


Fig 4. Distribution along the different *spa* types within A. MSSA; B. MRSA

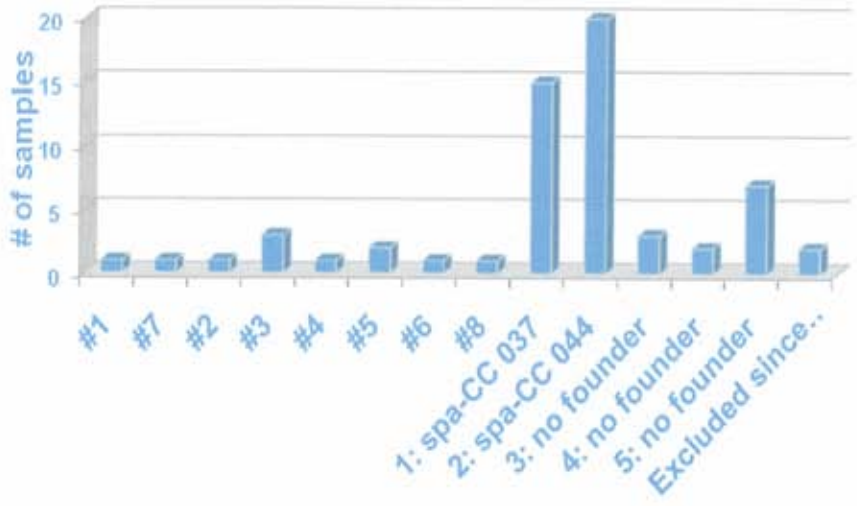


Fig 5:. Clustering *spa* types by BURP showing the number of samples belonging to each *spa*CC.

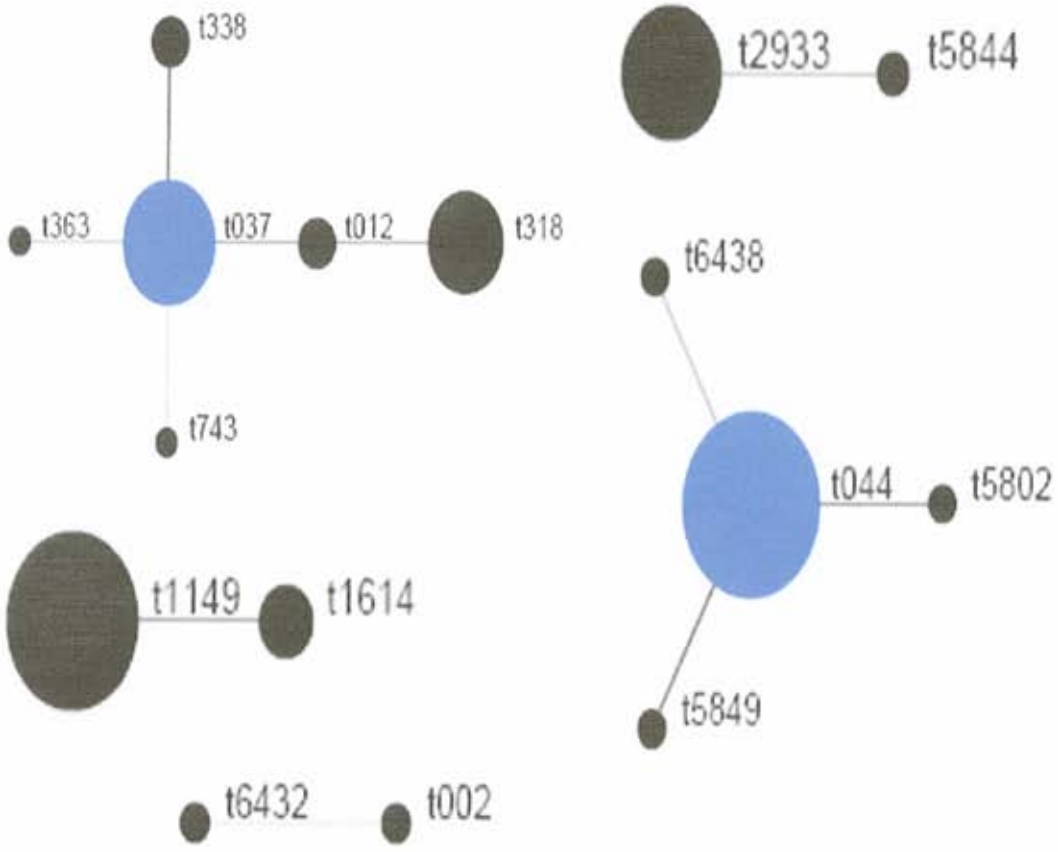


Fig 6: Clustering of the *spa* types as revealed by BURP. The blue circles represent the founder of each cluster. the size of each circle indicates the proportion of samples belonging to the *spa* type.

4.3 PFGE

Representative of each *spa* type 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 7).

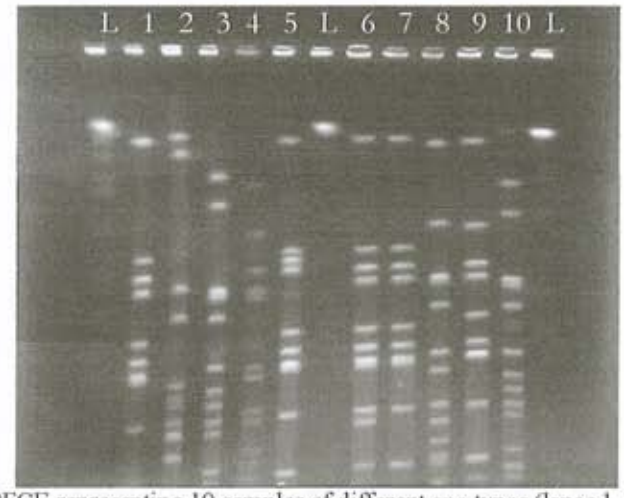


Fig 7. PFGE representing 10 samples of different *spa* types (lanes 1-10). L: BioRad ladder. *spa* type t044 lanes 1, 6, 7, 10, t6439 lane 2, t1149 lane 3, t701 lane 4, t5802 lane 5, t743 lane 8, t5849 lane 9.

In Fig 6 lanes 1, 6 ,7 and 10 represented isolates of *spa* type t044. Banding patterns when comparing lanes 6 to 7 were the same, while were different if compared to lanes 1 and 10. PFGE analysis also showed that isolates with different *spa* types could have similar banding patterns (lane 3: *spa* t1149 and lane 10: *spa* t044). Moreover, Fig 6 and 7 also reveal that with some isolates there was a positive correlation between PFGE and *spa* typing.

Fig 8. PFGE representing 5 samples of different *spa* types (lanes 1-5). L:BioRad ladder. *Spa* type t044 lanes 1,2,4, t1149 lane 3, t363 lane 5.

4.4 Biofilm Assay

S. epidermidis showed an optical density (OD) of 0.812 in the control well at 590 nm. This value did not vary significantly upon adding DNase and proteinase K. Sodium metaperiodate addition changed the OD to 0.213. Some samples showed an OD value between 0.160 and 0.216, similar to that of the blank, in the control well. Significantly sample 51 showed an absorbance value of 0.893 similar to that of the positive control. The OD then dropped to 0.167 in the well to which proteinase K was added, indicating a tendency of this isolate to form biofilm through protein deposition (Table 2). Sample 51 as opposed to other strains showed a small colony variant phenotype. Small colony variants (SCVs) of *S. aureus* are persistent colonies that show slowly on the plate. They have been a major concern in antibiotic-resistant infections (Seifert et al. 2003).

Table 2. Determination of the OD at 590 nm using ELISA reader for the studied isolates before and after the addition of one of the following: DNase, ProteinaseK, and sodium Metaperiodate..

	OD at 590 nm								
Well	1	2	3	9	10	51	Positive	Negative	Blank
Tested Isolates	0.176	0.176	0.216	0.193	0.174	0.893	0.812	0.201	0.166
DNase	0.163	0.204	0.150	0.261	0.241	0.733	0.924	0.209	0.207
Proteinase K	0.162	0.150	0.158	0.145	0.199	0.167	0.975	0.188	0.180
Sodium metaperiodate	0.251	0.278	0.291	0.214	0.199	0.880	0.213	0.309	0.212

Chapter 5

DISCUSSION

Infections with MRSA are becoming a major health issue in the region, yet questions on the epidemiology of MRSA carriage and infection remain unanswered. Advances in molecular typing techniques have resulted in a strict understanding of the spread and evolution of MRSA worldwide (Deurenberg and Stobberingh 2008). This preliminary epidemiological study focuses on *spa* typing and PFGE discriminatory power in *S. aureus* typing in samples obtained from Jordan.

41 MRSA isolates were distributed over 18 *spa* types, as opposed to 14 *spa* types for the 19 MSSA isolates. This reflects a higher diversity in the genetic profile of MSSA compared to MRSA as reported by Grundmann et al. (2010). The most commonly occurring *spa* types were *spa* type t044, t037, t002, t008, and t030. The high frequency of t044 was in concordance with Vindel et al. (2009) whose study showed that this type is widely disseminated in Europe. Tokajian et al. (2010) revealed a 38% of isolates belonging to *spa* t044 and 5% for t037 among isolates form Lebanon, which was in harmony with the predominance of t044 (28%) and t037 (8%) among Jordanian samples in our study. *spa* types t008 and t078 were most frequent according to Mellman et al. (2008), knowing that none of the isolates in this study belonged to these *spa* types.

Tokajian et al. (2010) revealed that *spa* t044 was detected only within MRSA, which was the case in this study except for one MSSA isolate being of *spa* type t044. *spa* type 1149, being a common *spa* type within MSSA

(Ruimy et al. 2008), represented 16% of the MSSA isolates in this study, which was also detected within MRSA isolates. *spa* t044, 1149, 318, 159, 1614, 012, 701, occurred in both MRSA and MSSA isolates indicating the heterogeneity and the genetic relationship between both MSSA and MRSA populations.

Using BURP algorithm all *spa* types were clustered into 2 *spa* CC, 8 singletons, and 3 groups with no founders. Both *spa* CC (044 and 037) contained more than one *spa* type and were distributed within MSSA and MRSA. These overlapping clonal complexes among MRSA and MSSA show the extension of MRSA from MSSA through the acquisition of the SCC*mec* (Deurenberg and Stobberingh 2008).

PFGE was used for the investigation of MRSA and was compared with other molecular typing techniques methods in many studies (Trindade et al. 2003). It investigates many sites around the chromosome as opposed to only a defined region within the chromosome as in the case of *spa* typing (Cookson et al. 2007). The 60 isolates were analyzed by PFGE to better assess the discriminatory ability, precision, and comparative rate of genetic change of *spa* typing. All the samples were typeable even after continuous culturing. The PFGE banding patterns helped in revealing minor genetic variations; some isolates of the same *spa* type were further distinguished having different PFGE banding patterns (Fig. 6 and 7). This was in accordance with the study by Hallen et al. (2007), where samples belonging to the same clonal complex (*spa*CC 002) exhibited different PFGE banding patterns. The genetic diversity within MRSA sharing the same *spa*-type helps in better investigating outbreaks and potential transmission events. Despite the long time PFGE requires to obtain the results, it is still the ideal tool for typing in

epidemiologic studies for assessing the effectiveness of targeted-prevention programs (Charlebois et al. 2002).

The findings of this study also agreed with others and revealed (Ruppitsch et al. 2006; Mellmann et al. 2008), that *spa* typing had a high degree of reproducibility especially with the introduction of software algorithms, such as BURP which better reveals a fast evolutionary clock, since it detects sequence repeats found in the *spa* gene. In contrast to other band based molecular typing techniques, *spa* typing allows inter-laboratory exchange and sharing of information through international networks, such as SeqNet. However, the results of the study suggest that *spa* typing be used in combination with staphylococcal cassette chromosome *mec* typing and MLST since MLST is based on sequencing seven housekeeping genes that are known to accumulate changes slowly which will allow an easier detection of the pathogen evolutionary pattern.

Absorbance values of all of the 60 isolates were compared to those of *S. epidermidis* (positive control). Significantly, sample 51 which is a MSSA belonging to *spa* t955 and clustered as a singleton, showed an initial absorbance of 0.893 which is comparable to 0.812 (positive control). This value decreased to 0.213 upon sodium metaperiodate addition and remained high in the presence of proteinase K and DNase. Knowing that sodium metaperiodate, proteinase K, and DNase indicates the presence of polysaccharide intercellular adhesion (PIA), proteinaceous factors, and genomic eDNA, respectively, we can infer that this isolate is capable of forming biofilm through the deposition of polysaccharides (Seidl et al. 2008). It has been shown that MSSA are also capable of forming biofilms in a study

conducted by Smith et al. (2008), where 28% of isolates were found to form fully recognized biofilms.

The ability of *S. aureus* to form more pronounced biofilms is related to the isolation site. The potential biofilm former obtained in this study has been isolated from the skin. This was in harmony with the finding that *S. aureus* isolated from the skin had better ability to form completely recognized biofilms compared to those taken from other sites of the body (Smith et al. 2008). This might be explained by the tendency of *S. aureus* to use the biofilm formation advantage to occupy the unstable environment of the human skin, placing the host at risk if the skin was breached (Akiyama et al. 2003). It is noteworthy that this potential biofilm former was MSSA and appeared as an SCV; both in general being common features previously studied by others such as Von Eiff et al. (2005). The clinical relevance of SCVs has been unclear for a long time (Neut et al. 2007), yet many studies have revealed that SCVs are largely responsible for the evolution of infections such as chronic osteomyelitis (Von Eiff et al. 1997) and cystic fibrosis, caused by biofilms (von Eiff et al. 1997, Drenkard and Ausubel 2002, Seifert et al. 2003). Understanding the capability of isolates to form biofilms is a prerequisite to comprehend the mechanisms employed in biofilm formation in *S. aureus* and determine the mediator of the biofilm to reduce biofilm-mediated infections and mortality in patients carrying severe *S. aureus* infections (O'Neill et al. 2007).

In conclusion sequence-based *spa* typing can be used as a rapid screening test when MRSA outbreak is suspected in areas and hospitals. MLST another important typing approach can be used for long-term international epidemiological surveys as the gold standard of population

analysis. Thus, the proper use of appropriate methods facilitates effective epidemiological surveys. On the other hand, *spa* and MLST sequences and PFGE pattern database established in this study will serve as a basis for information for the long term evolutionary and epidemiological studies of local *S. aureus* recovered not only in Jordan, but also in neighboring countries including Lebanon. Systematic surveillance of both hospital and community isolates in Jordan and Lebanon together with measures designed to limit the spread are required.

Finally, we recommend the use of MLST and *mec* subtyping to be conducted on a larger number of isolates collected simultaneously from Jordan, Lebanon and if possible Syria to better understand how populations of MSSA and MRSA have and are co-evolving and to combine that in studying genes enhancing the virulence of this important human pathogen with particular attention being drawn on those having a SCV phenotype.

Chapter 6

CONCLUSION

- The majority of the isolates 68% were found to be methicillin resistant *S. aureus*, and 32% were methicillin sensitive *S. aureus*, indicating a significant extensive spread of MRSA in the Jordanian community.
- These clinical isolates that were obtained from the same hospital environment, exhibited a wide genetic variation.
- *Spa* typing revealed 25 different *spa* types with the most common being t044 and t037.
- PFGE resulted sometimes in different banding pattern for isolates having the same *spa* type and in somehow similar banding pattern for samples belonging to different *spa* types.
- Both techniques, *spa* typing and PFGE, are essential in any comprehensive epidemiological approach.
- A potential MSSA biofilm former was identified among these samples taken from a skin infection which formed an SCV.

Chapter 7

BIBLIOGRAPHY

Aires de Sousa, M., & de Lencastre, H. (2004). Bridges from hospitals to the laboratory: Genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunol Med Microbio., 40*, 101-111.

Akiyama, H., Hamada, T., Huh, W.-K., Yamasaki, O., Oono, T., Fujimoto, W. et al. (2003). Confocal laser scanning microscopic observation of glycocalyx production by *Staphylococcus aureus* in skin lesions of bullous impetigo, atopic dermatitis and pemphigus foliaceus. *Br J Dermatol, 148*, 526–532.

Baba-Moussa, L., Anani, L., Scheftel, J.M., Couturier, M., Riegel, P., Haıkou, N., et al. (2008). Virulence factors produced by strains of *Staphylococcus aureus* isolated from urinary tract infections. *J Hosp Infect, 68*, 32-38.

Bekeredjian-Ding, I., Inamura, S., Giese, T., Moll, H., Endres, S., Sing, A., et al. (2007). *Staphylococcus aureus* Protein A Triggers T Cell-Independent B Cell Proliferation by Sensitizing B Cells for TLR2 Ligands. *J Immunol, 178*, 2803-2812.

Berglund, C., Ito, T., Ikeda, M., Ma, X., So¨derquist, B., & Hiramatsu, K. (2008). Novel Type of Staphylococcal Cassette Chromosome *mec* in a Methicillin-Resistant *Staphylococcus aureus* Strain Isolated in Sweden. *Antimicrob Agents Ch, 52,* 3512–3516.

Boyle-Vavra, S., & Daum, R. (2007). Community-acquired methicillin-resistant Staphylococcus aureus: The role of Panton Valentine leukocidin. *Lab Invest, 87,* 3–9.

Charlebois, D., Bangsberg, R., Moss, J., Moore, R., Moss, R., Chambers, F., et al. (2002). Population-based community prevalence of methicillin-resistant *Staphylococcus aureus* in the urban poor of San Francisco. *Clin Infect Dis, 34,* 425–433.

Chmelnitsky, S., Navon-Venezia, A., Leavit, E., Somekh, G., Regev-Yochai, M., Chowers, P., et al. (2007). SCC*mec* and *spa* types of methicillin-resistant *Staphylococcus aureus* strains in Israel. Detection of SCC*mec* type V. *Eur J Clin Microbiol Infect Dis*, 27, 385–390.

Cookson, B., Robinson, A., Monk, A., Murchan, S., Deplano, A., de Ryck, R., et al. (2007). Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* (MRSA) - the HARMONY collection. *J Clin Microbiol, 48.*

Cooper, J., & Feil, E. (2004). Multilocus sequence typing – what is resolved? *Trends Microbio, 12.*

Corrigan, R., Rigby, D., Handley, P., & Foster, T. (2007). The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. *Microbiol, 153,* 2435–2446.

Cucarella, C., Tormo, M., U′ beda, C., Trotonda, P., Monzo´n, M., Peris, C., et al. (2004). Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus. Infect Immun, 72,* 2177–2185.

Decker, C. (2008). Pathogenesis of MRSA Infections. *Dis. Mon.*, 54, 774-777.

Deurenberg, R., & Stobberingh, E. (2008). The evolution of *Staphylococcus aureus. Infect Genet Evol, 8*, 747-763.

Dinges, M., Orwin, P., & Schlievert, P. (2000). Exotoxins of *Staphylococcus aureus. Clin Microbiol Rev, 13*, 16-34.

Drenkard, E., & Ausubel, F M. (2002). Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature, 416, 740-743.

Fournier, B., & Hooper, DC., (2000). A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *J Bacteriol, 182,* 3955–3964.

Gómez, M., Lee, A., Reddy, B., Muir, A., Soong, G., Pitt, A., et al. (2004). Staphylococcus aureus protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat Med, 10,* 842 – 848.

Grundmann, H., Aanensen, D., Van den Wijngaard, C., Spratt, B., Dag Harmsen, D., Friedrich, A., & the European Staphylococcal Reference Laboratory Working Group. (2010). Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: A molecular-epidemiological analysis. *PLoS Med, 7.*

Hallin, M., Denis, O., Deplano, A., De Mendonca, R., De Ryck, R., Rottiers, S. et al. (2007). Genetic relatedness between methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*: Results of a national survey. *J Antimicrob Chemother, 59,* 465–472.

Heyn, B., Le Moal, M., Armand-Lefevre, L., & Nicolas-Chanoine, M.H. (2002). Multilocus sequence typing (MLST) shows that the "Iberian" clone of oxacillin-resistant *Staphylococcus aureus* has spread to France and acquired reduced susceptibility to teicoplanin. *J Antimicrob Chemother, 50,* 323–329.

Ito, T., Okuma, K., Ma, X., Yuzawa, H., & Hiramatsu, K. (2003). Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: Genomic island SCC. *Drug Resist Update, 6,* 41–52.

Izano, E., Amarante, M., Kher, W., & Kaplan, J. (2008). Differential roles of poly-*N*-Acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl Environ Microbiol, 74,* 470–476.

JiYeon, K. (2009). Understanding the evolution of methicillin-resistant *Staphylococcus aureus. Clin Microbiol Newslett, 31.*

Kahl, B.C., Mellmann, A., Deiwick, S., Peters, B., & Harmsen, R. (2005). Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent *mec*hanisms of genetic change in *Staphylococcus aureus. J Clin Microbiol, 43,* 502–505.

Khandavilli, S., Wilson, P., Cookson, B., Cepeda, J., Bellingan, G., & Brown, J. (2008). Utility of spa typing for investigating the local epidemiology of MRSA on a UK intensive care ward. *J Hosp Infect, 71,* 29-35.

Kikuchi, K., Takahashi, N., Piao, C., Totsuka, K., Nishida, H., & Uchiyama, T. (2003). Molecular epidemiology of methicillin-resistant

Staphylococcus aureus strains causing neonatal toxic shock Syndrome-like exanthematous disease in neonatal and perinatal wards. *J Clin Microbio., 41.*

Klevens, M., Morrison, M., Nadle, J., Petit, S., Gershman, K., Ray, S., et al. (2007). Invasive Methicillin-Resistant *Staphylococcus aureus* infections in the United States. *JAMA, 298,*1763-1771.

Kuhn, G., Francioli, P., & Blanc, D.S. (2007). Double-locus sequence typing using clfB and spa, a fast and simple method for epidemiological typing of methicillin-resistant *Staphylococcus aureus. J Clin Microbiol, 45,* 54–62.

Kuroda, M., Nagasaki, S., & Ohta, T. (2007). Sesquiterpene farnesol inhibits recycling of the C_{55} lipid carrier of the murein monomer precursor contributing to increased susceptibility to β-lactams in methicillin-resistant *Staphylococcus aureus. J Antimicrob Chemother, 59,* 425–432.

Ladhani, S., Joannou, C., Lochrie, D., Evans, R., & Poston, S. (1999). Clinical, microbial, and biochemical aspects of the exfoliative toxins causing Staphylococcal scalded-skin syndrome. *Clin Microbiol Rev, 12,* 224-24.

Levy, S., & Marshall, B. (2004). Antibacterial resistance worldwide: Causes, challenges and responses. *Nat Med Suppl, 10,* 122-129.

Lina, G., Piémont, Y., Godail-Gamot, F., Bes, M., Peter, MO., Gauduchon, V, et al. (1999). Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis, 29,* 1128-1132.

Luong, T., Newell, S., & Lee, C. (2003). mgr, a novel global regulator in *Staphylococcus aureus. J Bacteriol, 185,* 3703-3710.

Ma, X.X., Ito, T., Tiensasitorn, C., Jamklang, M., Chongtrakool, P., Boyle-Vavra, S., et al. (2002). Novel type of staphylococcal cassette chromosome *mec* identified in community-acquired methicillin-resistant *Staphylococcus aureus* strains. *Antimicrob Agents Chemother, 46,* 1147–1152.

Mackey-Lawrence, N., Potter, D., Cerca, N., & Jefferson, K. (2009). *Staphylococcus aureus* immunodominant surface antigen B is a cell-surface associated nucleic acid binding protein. *BMC Microbiol, 9,* 61.

Manna, A., & Cheung, A.L. (2001). Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. *Infect Immun, 69,* 885–896.

Manna, A., Ingavale, S., Maloney, M., van Wamel, W., & Cheung, A. (2004). Identification of sarV (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*. *J Bacteriol, 186*, 5267–5280.

Martin, F., Gomez, F., Wetzel, F., Memmi, G., O'Seaghdha, M., Soong, G., et al. (2009). *Staphylococcus aureus* activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. *J Clin Invest, 119*, 1931–1939.

McClure, J., Conly, J., Lau, V., Elsayed, S., Louie, T., Hutchins, W., et al. (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine Leukocidin genes and simultaneous discrimination of methicillin-susceptible from - resistant staphylococci. *J Clin Microbiol, 44*, 1141-1144.

McDonald, M., Dougall, A., Holt, D., Huygens, F., Oppedisano, F., Giffard, P.M., et al. (2006). Use of single-nucleotide polymorphism gentotyping system to demonstrate the unique epidemiology of methicillin-resistant *Staphylococcus aureus* in remote Aboriginal communitites. *J Clin Microbiol, 44*, 3720-3727.

McDougal, LK., Steward, CD., Killgore, GE., Chaitram, JM., McAllister, SK., & Tenover, FC. (2003). Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: Establishing a national database. *J Clin Microbiol, 41,* 5113-5120.

McNamara, P.J., Milligan-Monroe, K.C., Khalili, S., & Proctor, R.A. (2000). Identification, cloning, and initial characterization of rot, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. *J Bacteriol, 182,* 3197–3203.

Mellmann, A., Weninger, T., Berssenbrugge, C., Kechevoet, U., Friedrich, A.W., et al. (2008). Characterization of clonal relatedness among the natural population of *Staphylococcus aureus* strains by using *spa* Sequence typing and the BURP (Based upon repeat patterns) algorithm. *J Clin Microbiol, 46,* 2805-2808.

Merlin, MA., Wong, ML., Pryor, PW., Rynn, K., Marques-Baptista, A., Perritt, R., et al. (2009). Prevalence of methicillin-resistant *Staphylococcus aureus* on the stethoscopes of emergency medical services providers. *Prehosp Emerg Care., 13,* 71-74.

Milheiric'o, C., Oliveira, D., & de Lencastre, H. (2007). Update to the Multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. *Antimicrob Agents Ch, 51,* 3374–3377.

Murphy, J., Walshe, R., & Devocelle, M. (2008). A computational model of antibiotic-resistance mechanisms in methicillin-resistant *Staphylococcus aureus* (MRSA). *J Theor Biol, 254,* 284– 293.

Narukawa, M., Yasuoka, A., Note, R., & Funada, H. (2009). Sequence-based *spa* typing as a rapid screening method for the areal and nosocomial outbreaks of MRSA. *Tohoku J Exp Med, 218,* 207-213.

Neut, D., van der Mei, H., Bulstra, S., & Busscher, H. (2007). The role of small-colony variants in failure to diagnose and treat biofilm infections in orthopedics. *Acta Orthopaedica, 78,* 299–308.

O'Gara, JP. (2007). *ica* and beyond: Biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol Lett, 270,* 179–188.

Oliveira, D.C., Milheirico, C., & de Lencastre, H. (2006). Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCCmec yype VI, *Antimicrob Agents Chemother, 50,* 3457–3459.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, A., Loughman, A., et al. (2008). A Novel *Staphylococcus aureus* biofilm

phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *J. Bacteriol., 190,* 3835–3850.

O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.R. et al. (2007). Association between methicillin susceptibility and biofilm regulation in *Staphylococcus aureus* isolates from device-related infections. *J Clin Microbiol, 45,* 1379–1388.

Otto, M. (2008). Targeted immunotherapy for staphylococcal infections: Focus on anti-MSCRAMM antibodies. *BioDrugs, 22,* 27–36.

Patel M., Hoesley, C., Moser, S., Stamm, A., Baddley, J., &Waites, K. (2008). Dissemination of community-associated methicillin-resistant *Staphylococcus aureus* in a tertiary care hospital. *Southern Med J, 101.*

Patou, J., Gevaert, P., Van Zele, T., Holtappels, G., Cauwenberge, P., & Bachert, C. (2008). Staphylococcus aureus enterotoxin B, protein A, and lipoteichoic acid stimulations in nasal polyps. *J Allergy Clin Immunol, 121,* 110-115.

Recsei, P., Gruss, A., & Novick, R. (1987). Cloning, sequence, and expression of the lysostaphin gene from *Staphylococcus simulans. Proc Natl Acad Sci USA, 84,* 1127–1131.

Ruimy, R., Maiga, A., Armand-Lefevre, L., Maiga, I., Diallo, A., Koumaré, et al. (2008). The carriage population of *Staphylococcus aureus* from

Mali is composed of a combination of pandemic clones and the divergent Panton-Valentine Leukocidin-positive genotype ST152. *J. Bacteriol. 190*, 3962-3968.

Ruppitsch, W., Indra, A., Stöger, A., Mayer, B., Stadlbauer, S., Wewalka, G., et al. (2006). Classifying spa types in complexes improves interpretation of typing results for methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol, 44*, 2442-2448.

Sandel, M.K., & McKillip, J.L. (2004). Virulence and recovery of *Staphylococcus aureus* relevant to the food industry using improvements on traditional approaches. *Food Control, 15*, 5-10.

Schmidt, K.A., Manna, A.C., Gill, S., & Cheung, A.L. (2001). SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus*. *Infect. Immun., 69*, 4749–4758.

Schouls, LM., Spalburg, EC., van Luit, M., Huijsdens, XW., Pluister, GN., van Santen-Verheuvel, MG., et al. (2009). Multiple-locus variable number tandem repeat analysis of *Staphylococcus aureus*: Comparison with pulsed-field gel electrophoresis and spa-typing. *PLoS One, 4*.

Seidl, K., Goerke, C., Wolz, C., Mack, D., Berger-Bächi, B., & Bischoff, M. (2008). *Staphylococcus aureus* CcpA affects biofilm formation. *Infect Immun, 76,* 2044-2050.

Seifert, H., Wisplinghoff ,H., Schnabel, P., & von Eiff, C.(2003). Small colony variants of *Staphylococcus aureus* and pace maker related infection. *Emerg Infect Dis, 9,* 1316-1318.

Silverman, GJ., & Goodyear, CS. (2006). Confounding B-cell defences: Lessons from a staphylococcal superantigen. *Nat Rev Immunol, 6,* 465-475.

Smith, K., Perez, A., Ramage, G., Lappin, D., Gemmell, C., & Lang, S. (2008). Biofilm formation by Scottish clinical isolates of *Staphylococcus aureus. J Med Microbiol, 57,* 1018-1023.

Tegmark, K., Karlsson, A., & Arvidson, S. (2000). Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus. Mol Microbiol, 37,* 398–409.

Tenover, F., McDonald, C., & Clifford, L. (2005). Vancomycin-resistant staphylococci and enterococci: Epidemiology and control. *Curr Opin Infect Dis, 18,* 300-305.

Te Witt, R., Kanhan, V., & Van Leeuwen, W. (2009). Comparison of the DiversiLab™ system, pulsed-field gel electrophoresis and multi-locus

sequence typing for the characterization of epidemic reference MRSA strains. *J Microbiol Meth, 77,* 130-133.

Todar, K. (2008). Todar's online textbook of bacteriology. [n.p.]: [n.p.]. Retrieved 15 June, 2010 from: http://www.textbookofbacteriology.net

Tokajian, S., Abou Khalil, P., Jabbour, D., Rizk, M., Farah, M., Hashwa, F., & Araj, G. (2010). Molecular characterization of *Staphylococcus aureus* in Lebanon. *Epidemiol Infect, 138,* 707-712.

Tormo, M., U´ beda, C., Martı, M., Maiques, E., Cucarella, C., Valle, J., et al. (2007). Phase-variable expression of the biofilm-associated protein (Bap) in *Staphylococcus aureus*. *Microbiol, 153,* 1702–1710.

Trindade, P., McCulloch, J., Oliveira, G., & Mamizuka, E. (2003). Molecular techniques for MRSA typing: Current issues and perspectives. *Braz J Infect D, 71,* 32-43.

Urwin, R., & Maiden, MC. (2003). Multi-locus sequence typing: A tool for global epidemiology. *Trends Microbiol, 10,* 479-487.

Von Eiff, C., Friedrich, A., Becker, K., & Peters, G. (2005). Comparative in vitro activity of Ceftobiprole against Staphylococci displaying normal and small-colony variant phenotypes. *Antimicrob Agents Chemother, 49,* 4372-4374.

Von Eiff, C., Bettin, D., Proctor, R A., Rolauffs, B., Lindner, N., Winkelmann, W., et al. (1997). Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clin Infect Dis, 25,* 1250-1251.

Yarwood, J. M., McCormick, J. K., & Schlievert, P.M. (2001). Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus. J Bacteriol, 183,* 1113-1123.

Zetola, N., Francis, J., Nuermberger, E., & Bishai, W. (2005). Community-acquired meticillin-resistant *Staphylococcus aureus*: An emerging threat. *Lancet Infect Dis, 5,* 275–86

Zhang, K., McClure, J.-A., Elsayed, S., & Conly, J. M. (2009). Novel Staphylococcal Cassette Chromosome mec Type, Tentatively Designated Type VIII, Harboring Class A mec and Type 4 ccr Gene Complexes in a Canadian Epidemic Strain of Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother, 53,* 531-540.