Master Program in Clinical Laboratory Science

# Molecular characterization of methicillin-resistant *Staphylococcus aureus* strains isolated from three Arab countries

# المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) المعزولة من ثلاثة بلدان عربية بواسطة تقنيات الأحياء الجزيئية

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# DEDICATION TO

I dedicate this work to my father Nizar and my mother Amal for inspiring me to continuously seek knowledge and strife for improvement. A special word of gratitude to my dear wife Manal and my lovely daughters Aseel and Raneem for endurance, support and understanding during the time I spend to complete this work. I am also grateful to my brothers Ameed and Muhannad for their actual support and encouragement.

I am greatly indebted to them all, without them this thesis might not have been fulfilled.

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| | |
|---|---|
| **MSSA** | Methicillin Susceptible *Staphylococcus aureus* |
| **MRSA** | Methicillin Resistant *Staphylococcus aureus* |
| **SCC*mec*** | Staphylococcal Cassette Chromosome *mec* |
| **VRSA** | Vancomycin resistant *S. aureus* |
| **PFGE** | Pulsed Field Gel Electrophoresis |
| **MLST** | Multilocus Sequence Typing |
| **HA-MRSA** | Hospital Acquired Methicillin Resistant *Staphylococcus aureus* |
| **CA-MRSA** | Community Aquired Methicillin Resistant *Staphylococcus aureus* |
| **PVL** | Panton-Valentine leukocidin |
| **TSS** | Toxic Shock Syndrome |
| **SAgs** | Super Antigen Toxins |
| **PBP** | Penicillin-Binding Protein |
| **TPase** | Trans Peptidase |
| **TGase** | Trans Glycosylase |
| **MIC** | Minimum Inhibitory Concentration |
| **UPGMA** | Unweighted Pair Group Matching Analysis |
| **spa** | Staphylococcal Protein A |
| **SSR** | Short Sequence Repeat |
| **NCCLS** | National Committee for Clinical Laboratory Standards |
| **RFT** | Restriction Fragment Typing |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **ST** | Sequence Typing |
| **AMK** | Amikacin |
| **CAZ** | Ceftazidime |
| **CIP** | Ciprofloxacin |
| **CLI** | Clindamycin |
| **CTX** | Cefotaxime |
| **ERY** | Erythromycin |
| **FUS** | Fusidic acid |
| **GEN** | Gentamicin |
| **IMP** | Imipenem |
| **SXT** | Sulfamethoxazole-trimethoprim |
| **TPL** | Teicoplanin |
| **VAN** | Vancomycin |
| **OXA** | Oxacillin |
| **PEN** | Penicillin**.** |
| **MDR** | Multidrug resistance |
| **SPSS** | Program Statistical Package for Social Sciences |

# ABSTRACT

The aim of this study was to utilize molecular techniques for the development of rapid molecular methods to detect methicillin resistant *Staphylococcus aureus* (MRSA) and subsequently to study important antibiotic determinants and molecular characterize MRSA isolates from three different Arab countries. Sixty seven clinical MRSA isolates were collected. Seventeen from West Bank of Palestine, 25 from Jordan, and 25 from Iraq. Conventional oxacillin disc diffusion test and PCR-based hybridization assay (Geno Type MRSA Direct) were used for simultaneous identification and detection of methicillin resistance gene to enable rapid identification of MRSA. All sixty seven strains (100%), identified as methicillin resistant phenotypically by conventional oxacillin disc were also positive by Geno Type MRSA assay specific for the *mec*A gene.

The prevalence of oxacillin, penicillinG, erythromycin, clindamycin, ciprofloxacin, gentamicin, cefotaxime, fusidic acid, ceftazidime and sulfamethoxazole-trimethoprim resistance among MRSA isolates was above 82%. Resistance rates of MRSA to other antibiotics were as follows: 64.2% resistant to each of amikacin and imipenem, 13.4% to teicoplanin. Vancomycin-resistant isolates were not observed in this study. Resistance to oxacillin/ penicillinG/ erythromycin/ clindamycin/ ciprofloxacin/ gentamicin/ cefotaxime/ fusidic acid/ amikacin/ imipenem/ ceftazidime/ sulfamethoxazole-trimethoprim was the most common pattern of multiple resistance in the three participant countries.

Nucleotide comparison and maximum likelihood phylogenetic analysis revealed the existence of two main clusters of MRSA in these three countries: Cluster (I) comprised 18.2% (n=2) of the isolates, and contained one representative isolates from each of Jordan and Iraq. Cluster (II) comprised most of the strains 81.8% (n=9), and contained 5 isolates from Palestine, two isolates from Jordan and two from Iraq. Since the strain distribution in these two clusters is characteristic, it denotes the existence of two main clones.

Phylogenetic analysis showed that isolates of cluster II is identical to international strains that have the accession number X61307, AM407300,

U54636 and EF094528 belonging to France, Switzerland, Brazil and Australia, respectively.

These results suggest that cluster II strains are globally disseminated and often multi drug resistant, which poses a global imminent threat. Thus, further studies are needed for the surveillance of MRSA strains using a combination of molecular typing techniques, to relate our strains to international epidemic clones and to provide a much larger view of molecular epidemiology of MRSA isolates in the Arab world.

# الملخص

## دراسة المكورات العنقودية الذهبية المقاومة للميثيسيلين ( MRSA) المعزولة من ثلاثة مناطق مختلفة في العالم العربي بواسطة تقنيات الأحياء الجزيئية

هدفت الدراسة إلى استخدام التقنيات الجزيئية لتطوير طرق التشخيص السريرية للكشف عن المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) ومن ثم دراسة تأثير المضادات الحيوية على هذا النوع من البكتيريا. أجريت الدراسة على سبع و ستين عزلة مختلفة معزولة من ثلاثة مناطق في العالم العربي ( 17 من فلسطين ، 25 من الأردن ، و 25 من العراق). استخدمت طريقة اختبار الحساسية لقرص الأوكساسلين وتفاعل البلمرة المتسلسل على أساس فحص التهجين (PCR-based hybridization assay) لتحديد مقاومة هذه العزلات للميثيسيلين. أظهرت نتيجتي كلا الفحصين توافقا في النتائج بنسبة (100 ٪).

أظهرت الدراسة مقاومة العزلات بنسبة تزيد على 82 ٪ لكل من المضادات: الأوكساسلين، البنسلين، الاريثروميسين، الكليندامايسين، سيبروفلوكساسين، جنتاميسين، سيفوتاكسيم، حامض الفوسيديك، السيفتازيديم وسلفاميثوكسازول – ترايميثوبريم. وكانت نسبة مقاومة هذه الجرثومة للمضادات الحيوية الأخرى على النحو التالي : 64.2٪ لكل من الأميكاسين والايميبنام ، 13.4٪ لتيكوبلانين. وقد أظهرت جميع العزلات حساسة للمضاد فانكومايسين. أظهرت الدراسة ان نمط مقاومة العزلات المتعدد للمضادات الحيوية: الأوكساسلين/ البنسلين/ الاريثروميسين / الكليندامايسين / سيبروفلوكساسين / جنتاميسين / السيفوتاكسيم / حمض الفوسيديك / الأميكاسين / الايميبنام / السيفتازيديم / سلفاميثوكسازول – ترايميثوبريم كان سلفاميثوكسازول – ميثوبريم الأكثر شيوعا في البلدان الثلاث.

أظهرت مقارنة تسلسل الحمض النووي للجين المفترض (*spa*) للعزلات مع جينات مفترضة مشابهة للجين (*spa*) من حيث التسلسل والتي تم البحث عنها في شبكة المعلومات بواسطة الشجرة الجينية وجود مجموعتين رئيسيتين (I,II) في هذه البلدان الثلاثة، وان عزلات المجموعة الثانية مطابق لسلالات عالمية.

تشير هذه النتائج إلى أن المجموعة الثانية عالمية ومقاومة لعديد من المضادات الحيوية مما يشكل تهديدا وشيكا عالميا. وهناك حاجة إلى مزيد من الدراسات لمراقبة ودراسة سلالات هذه الجرثومة في العالم العربي ومقارنتها بالسلالات العالمية باستخدام طرق التقنيات الجزيئية المتعددة.

# Chapter One

# Introduction and literature review

## 1.1 Introduction

The prevalence of *Staphylococcus aureus* is of growing concern. Its strong adaptive power to antibiotics has resulted in the emergence of methicillin-resistant *S. aureus* (MRSA). Resistance to methicillin (oxacillin) and other β-lactam antibiotics is caused by the *mecA* gene, which is situated on a mobile genetic element, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Six SCC*mec* types (I to VI) have been distinguished (1). Likewise, the presence of *van*A leads to vancomycin resistant strains of *S. aureus* (VRSA) (2). Molecular typing approaches have been used to great advantage in identifying and monitoring the local and international spread of MRSA outbreak strains. These include pulsed-field gel electrophoresis (PFGE), multi locus sequence typing (MLST) and *spa* typing (3,4). Several MRSA clones have emerged and disseminated worldwide. Although, in the past, MRSA strains were mainly hospital- acquired (HA-MRSA), during the late 1990s, community-acquired MRSA (CA-MRSA) has emerged. CA-MRSA harbors SCC*mec* type IV or V and is often associated with Panton-Valentine leukocidin (PVL) (5).

## 1.2 Staphylococcus aureus

The name *Staphylococcus* comes from the Greek *staphyle*, meaning a bunch of grapes, and *kokkos*, meaning berry, and that is what Staph look like under the microscope, like a bunch of grapes or little round berries. *S. aureus* are gram-positive, facultative anaerobic, usually unencapsulated cocci. *Staphylococcus aureus* is a versatile human pathogen causing infections ranging from relatively mild involvement of skin and soft tissue to life- threatening sepsis, pneumonia, and toxic shock syndrome (TSS). The organism causes disease through production of numerous cell surfaces and secreted virulence factors, and disease is facilitated by its propensity to develop resistance to multiple antibiotics (6,7).

Before the availability of antibiotics, invasive infections caused by *S. aureus* were often fatal. The introduction of penicillin greatly improved the prognosis for patients with severe staphylococcal infections, but after few years of clinical use, resistance appeared owing to production of β-lactamases (8). Methicillin was designed to resist β-lactamase degradation, but MRSA strains that were resistant to all β-lactam antibiotics were identified soon after methicillin was introduced into clinical practice (8). Until recently, MRSA was predominantly a nosocomial pathogen causing hospital-acquired infections, but MRSA

strains are now being increasingly isolated from community-acquired infections as well.

Vancomycin is the drug of choice to treat infections caused by MRSA, but increase in vancomycin use has led to the emergence of vancomycin-resistant *S. aureus*.

Currently, MRSA can be categorized based on the settings in which patients acquire the infection: hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA) (9,10).

## 1.3 Epidemiology

### 1.3.1 Carriage

Although S. *aureus* can be cultured from multiple sites of the skin and mucosal surfaces of carriers, the primary reservoir of staphylococci is thought to be the anterior nares (11). Nasal carriage rates of 25 % - 40 % have been reported, and are known to be influenced by ethnicity, age, exposure to antibiotics and the hospital environment (12).

Carriage of S. *aureus* in the nose appears to play a key role in the epidemiology and pathogenesis of infection (13, 11). Additionally, nasal carriage of S. *aureus* has been identified as a risk factor for the development of infections in various settings (11).

Several studies suggested that MRSA carriage constitutes a greater risk for the development of S. *aureus* infection than does MSSA carriage.

This could be a result of drug resistance of MRSA compared with MSSA or of a more vulnerable category of patients being colonized by MRSA (11, 14, 15). Elimination of nasal carriage would theoretically reduce the infection rates in populations in which it has been identified as a risk factor (11).

### 1.3.2 Virulence factors

*S. aureus* harbors an extensive battery of virulence factors contributing to its ability to propagate and spread within the human host. These factors include cell surface components (e.g., protein A, fibronectin-binding protein, collagen-binding protein, clumping factor), and exoproteins (e.g., Enterotoxins, exfoliatins, toxic shock syndrome toxin, and Panton-Valentine leucocidin (PVL)(16).

Among the secreted virulence factors of S. *aureus* are the super antigen toxins (SAgs), which include TSS toxin 1 (TSST-1) and staphylococcal enterotoxin (SE) serotypes A to Q (SEA to SEQ), excluding F. These toxins cause Toxic Shock Syndrome (TSS) and related illnesses through their capacity to induce massive cytokine release from both macrophages and T cells (17).

### 1.3.3 Mode of transmission

S. *aureus* including MRSA strains are primarily spread from patient to patient via the transiently colonized hands of health-care workers during patient contact or handling contaminated materials (18, 19). Environmental surfaces are not thought to play a major role in transmission except in special populations such as patients in burn units or intensive care units. Airborne transmission of S. *aureus* has been reported but does not appear to be an important mode of spread, except possibly in burn units (19).

The cohort of colonized or infected MRSA patients usually constitutes the most significant in-hospital reservoir from which MRSA is transmitted to other individuals (18). Because spread of MRSA in health care settings is often clonal, hand hygiene and barrier precautions are often effective in interrupting its spread. Targeted surveillance for MRSA is also a useful aid for infection control (20).

### 1.3.4 Laboratory diagnosis

*Staphylococcus aureus* is gram positive cocci in clusters; it is catalase and coagulase positive, facultative anaerobe that shows beta hemolysis on sheep blood agar (21).

Laboratory diagnosis of S. *aureus* is based on phenotypic characteristics and biochemical tests: typical colony morphology appears

as large yellow or grey colonies, coagulase positive, ferments mannitol and the produces of heat stable nucleases. Coagulase production is considered the most reliable characteristic for identifying *S. aureus.* A four-hour tube coagulase test is a definitive test. A slide test is used as a screening method for the detection of the clumping factor. Latex agglutination tests are also used as rapid identification tests (21).

### 1.3.5 Treatment

Most strains of *S. aureus* are treated with penicillin -type antibiotics, such as flucloxacillin, cloxacillin, dicloxacillin and methicillin. Alternative antibiotics, such as erythromycin, may be used in individuals allergic to penicillin.

Treatment of MRSA infections remains difficult due to multiple resistance to antibiotics. Vancomycin is the drug of choice but should be the last option. Teicoplanin and daptomycin are two investigational antibiotics related to vancomycin in structure and in spectrum of activity (8). Mild to moderately severe MRSA infections may be treated with trimethoprim-sulfamethoxazole or minocycline if the organism is susceptible to these agents. Although most strains are currently susceptible to rifampin and fusidic acid, these agents should not be used alone because of the risk for selecting resistant mutants during treatment(19).

## 1.4 Clinical Manifestations

### 1.4.1 Hospital acquired MRSA

The increased incidence of multi drug-resistant *S. aureus* strains among nosocomial or hospital-acquired (HA) infections has added a challenging dimension to the *S. aureus* problem. These strains are typically labeled HA methicillin- resistant *S. aureus* (MRSA) strains or simply MRSA strains. Several risk factors, such as recent hospitalization or exposure to a health care setting, residence in long-term-care facilities, invasive or surgical procedures, and intravenous drug use, predispose a patient to MRSA acquisition (22)

Nosocomial MRSA (HA) infections represent a burden for both patients and health care systems, because of their association with high morbidity and mortality and increased hospitalization costs. Recent data from the Center for Disease Control and Prevention showed that 59.5% of all health care–associated *S. aureus* infections in the United States are caused by MRSA (23).

Since its initial identification in 1961, many risk factors for HA-MRSA infection have been recognized. These include bacteremia, pneumonia, surgical site infections, and other nosocomial infections (24).

### 1.4.2. Community-acquired MRSA strains

In the community, the majority of *S. aureus* infections, which include skin and soft tissue infections (17), are caused by methicillin-susceptible *S.*aureus (MSSA) strains. However, since 1991 there have been increasing reports of MRSA infections in the community and in patients with and without risk factors for MRSA infection. These MRSA strains, commonly referred to as community- associated MRSA (CA-MRSA) (25, 17).

### 1.4.3 CA MRSA vs. HA MRSA

Community-acquired strains of MRSA are distinct from HA-MRSA strains from genotypic, and epidemiologic perspectives (22).

Genetically, CA-MRSA strains possess several features that distinguish them from HA-MRSA. Although a small percentage of CA-MRSA contain SCC*mec* type V, these strains predominantly carry SCC*mec* type IV, which is smaller in size than the gene cassette of HA-MRSA (types I, II, and III) (26). This observed differential in SCC size may allow for more efficient transfer of resistance among different bacteria, a factor that may be relevant in the alarmingly rapid emergence of CA-MRSA.

The potential of CA-MRSA strains to cause serious illness is further underscored by their production of a relatively greater number of

recognized staphylococcal virulence factors compared with HA-MRSA. Most notably, CA-MRSA strains frequently carry the Panton-Valentine leukocidin genes that produce cytotoxins associated with tissue necrosis and leukocyte destruction (27).

In contrast to HA-MRSA, CA-MRSA strains are commonly susceptible to the majority of other non-β-lactam anti staphylococcal antibiotics and have a common pulsed- field gel electrophoresis (PFGE) pattern (4). In the USA, two lineages, namely USA400 according to the PFGE pattern and USA300, are the most frequent and widely disseminated CA-MRSA (23).

## 1.5 Antibiotic resistance in *S. aureus*

### 1.5.1 Methicillin resistance

The extensive resistance of methicillin-resistant *S. aureus* to all *β*-lactam antibiotics is related to the properties of the key component of this resistance mechanism: the "acquired" penicillin-binding protein PBP-2a, which has unusual low affinity for all *β*-lactam antibiotics (28, 29).

PBP2a is encoded by the *mec*A gene, which is carried by a large mobile genetic element that is designated staphylococcal cassette chromosome *mec* (SCC*mec*) and that is integrated into the chromosomes of MRSA strains isolated from hospitals in various countries throughout the world (25, 28, 30, 31).

Based on homologies of PBP2a with other PBPs, PBP2a was suggested to have both trans peptidase (TPase) and trans glycosylase (TGase) activity (32), although neither of these activities was proven, an attachment TPase activity attributed to PBP2a in methicillin-resistant *Staphylococcus epidermidis* has been demonstrated (33).

PBPs are involved in the assembly of the bacterial cell-wall peptidoglycan. The strategy of β-Iactam resistance in MRSA involves the addition of the new, acquired PBP2a to the complement of the four native staphylococcal PBPs. The low-affinity PBP2a is assumed to take over the cell wall biosynthetic functions of normal PBPs in the presence of β-lactam antibiotics, which rapidly inactivate each of the four native staphylococcal PBPs at concentrations that are far below the minimum needed to inhibit the growth of most MRSA strains (28, 29).

Inactivation of the transglycosylase domain, but not the transpeptidase domain, of PBP2 of *S. aureus* prevents expression of β-lactam resistance, despite the presence of the low-affinity PBP2a. The observations suggest that cell-wall synthesis in the presence of β-lactam antibiotics requires the cooperative functioning of the transglycosylase domain of the native staphylococcal PBP2 and the transpeptidase domain of the PBP2a, a protein imported by *S. aureus* from an extra species source (28).

Phase-contrast microscopy study by G. Prevost and colleges (34) showed that cells swelled at low concentrations of β-lactam antibiotics in both MRSA and variants which had lost methicillin resistance. Cells of variants which had lost methicillin resistance were lysed easily when higher concentrations of antibiotic were used. In contrast, MRSA cells remained swollen at even higher concentrations of antibiotics. Furthermore, bacterial growth was inhibited at antibiotic concentrations much lower than MICs for MRSA. Examination of the penicillin-binding proteins (PBPs) in MRSA revealed that a new PBP2a was induced in large quantity by exposure to beta-lactams. PBP2a was produced constitutively in variants of MRSA which had lost a penicillinase plasmid (28, 33, 35).

The induction of PBP2a by β-lactams was not detected in variants which had lost methicillin resistance. High concentrations of *β*-lactam were required for saturation of PBP2a. The optimum antibiotic concentration for the induction of PBP2a varied with the *β*-lactam used as the inducer, and PBP2a was produced in a larger amount at 32°C than at 35°C. From these results, it suggested that the mechanism of methicillin resistance depends on the induction of PBP2a, which may function as an alternative route enzyme for PBP2 or PBP3 or may be a particular enzyme involved in peptidoglycan synthesis. (33, 34, 36)

### 1.5.2 Vancomycin resistance

Vancomycin is the last drug of choice for therapy of infections due to methicillin (oxacillin)-resistant *S. aureus* (MRSA), but increase in vancomycin use has led to the emergence of two types of glycopeptide-resistant *S. aureus*. The first one, designated vancomycin intermediate-resistant *S. aureus*, is associated with a thickened and poorly cross-linked cell wall, resulting in accumulation of acyl-D-alanyl-D-alanine (X-D-Ala-D-Ala) targets in the periphery that sequester glycopeptides (38.) The second type, vancomycin-resistant *S. aureus* (VRSA), is due to acquisition from *Enterococcus* spp. of the *vanA* operon, carried by transposon Tn*1546*, resulting in high-level resistance (37, 38).

## 1.6 The Staphylococcal accessory genome

### 1.6.1 The staphylococcal cassette chromosome *mec* (SCC*mec*)

The SCC*mec* element is present in five (I to V) different allotypes and contains a characteristic combination of two essential genetic components, the *mec* gene complex and the *ccr* gene complex (39). The SCC*mec* elements are widely disseminated among coagulase-negative staphylococci, and studies have presented evidence for horizontal transfer between staphylococcal species, although this seems to be a rare event(40).

Although alteration of target penicillin-binding proteins is the primary mechanism of resistance to *β*-lactam antibiotics, over the years MRSA strains have gained multiple mechanisms of resistance to several classes of antimicrobials, including macrolides, aminoglycosides, f1uoroquinolones, tetracyclines, and lincosamide antibiotics such as clindamycin. For the past several decades, glycopeptide antibiotics, such as vancomycin, have been considered the only agents to which MRSAs have not developed resistance (24). Unfortunately, due to over use of glycopeptide antibiotics, MRSAs have emerged with reduced susceptibility to these agents as well (41).

The central genetic component of the resistance mechanism in these bacteria is *mec*A, which-embedded in a larger block of "foreign" DNA-is not native to *S. aureus*, but was imported from an as yet unidentified extra species source (42).

The *mec*A gene is harbored in a large mobile genetic element referred to as the staphylococcal chromosomal cassette *mec* (SCC*mec*) that has a unique chromosomal integration locus (39, 43). SCC*mec* is a mobile genetic element characterized by the presence of terminal inverted and direct repeats, a set of site-specific recombinase genes (*ccrA* and *ccrB*), and the *mecA* gene complex Figure 1.1 (39, 43). SCC is a basic mobile genetic element that serves as the vehicle for gene exchange among staphylococcal species (39)

The *ccr* gene complex contains two site-specific recombinase genes, ccrA and *ccrB,* which are responsible for the mobility of SCC*mec* (25, 30). SCC*mec* may be classified into four major types according to the class of *mecA* gene complex and the type of *ccr* gene complex present (30): type I, class B and *ccr*AB1; type II, class A and *ccr*AB2; type III, class A and *ccr*AB3; and type IV, class B and *ccr*AB2. The region other than the *mec* and *ccr* gene complexes is designated the J (junkyard) region (30, 39, 42).

Three relatively large SCC*mec* elements, types I, II, and III, were initially described for MRSA isolates, which were mostly obtained from patients frequenting health care environments. SCC*mec* types I, II, and III are distinguished on the basis of their sizes, which range from 26 and 67 kb, and genetic compositions, in which their genomes include recombinases and antibiotic resistance genes. (22, 30)

A smaller SCC*mec* element, type IV, was first identified in community-acquired MRSA (CA-MRSA) isolates from Chicago (25) and was subsequently found in MRSA isolates from other geographic locales(1).

More recently, a smaller fourth *mec* element, SCC*mec* type IV (20 to 24 kb), was independently identified among representatives of the Pediatric clone and in two community-acquired MRSA strains (25,44) and the newest member, type V. (39)

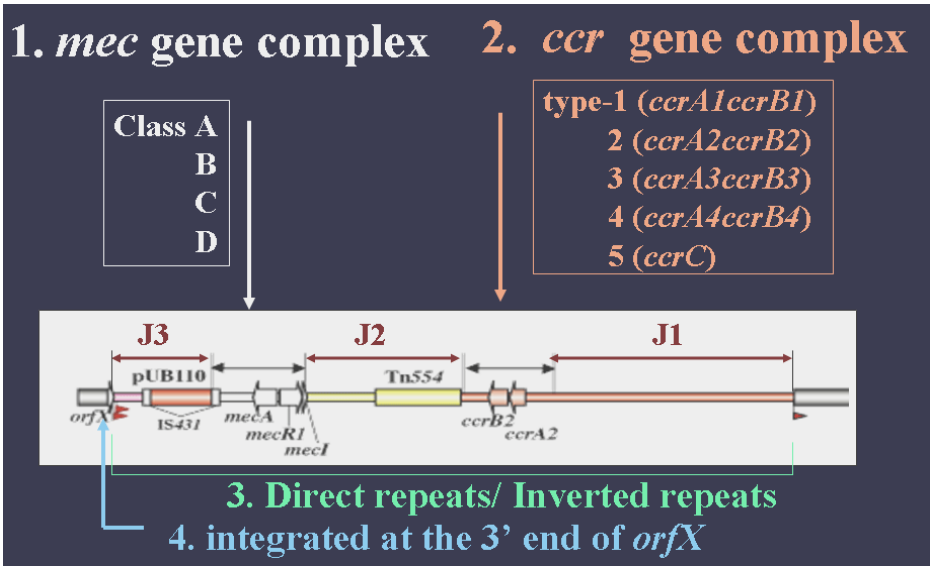


**Figure 1.1: The essential structure of SCC*mec* Elements**

### 1.6.2 Panton-Valentine Leucocidin (PVL)

The toxic effect of PVL, results from the synergistic action of two separate exoproteins, namely, LukS-PV and LukF-PV. These proteins are encoded by two contiguous and cotranscribed genes (LukS-PV and LukF-PV), which are carried on temperate bacteriophages (45, 46).

PVL is a pore-forming cytotoxin that targets human and rabbit mononuclear and polymorphonuclear cells. Most notably, CA-MRSA strains frequently carry the Panton-Valentine leukocidin genes that produce cytotoxins associated with tissue necrosis and leukocyte destruction, although argument remains concerning the definitive role of these genes in CA-MRSA (22).

## 1.7. Molecular Techniques for MRSA Typing:

The increase frequency of MRSA and the possibility of emergence of resistance to vancomycin demands a reliable characterization of isolates and identification of clonal spread of MRSA. Enough information must be generated to permit the implementation of appropriate measures for control of infection, so that outbreaks can be contained. The most commonly used molecular typing techniques include: pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and *spa* typing (47).

PFGE became the "gold standard" for *S. aureus* strain typing mainly because of its excellent discriminatory power, especially for analysis of the local short-term epidemiology. PFGE is based on the digestion of chromosomal DNA with the restriction enzyme *Sma*I, followed by agarose gel electrophoresis. The PFGE patterns are analyzed with a software package with Dice comparison and unweighted pair group matching analysis (UPGMA) settings according to the criteria of Tenover e*t al*. (48). Much effort was put into the standardization of protocols and the interpretation of PFGE patterns (49); nevertheless, interpretation of the results is hampered by a lack of both inter laboratory reproducibility and a common nomenclature (49).

MLST is based on the sequencing of seven “housekeeping” genes, allows more precise identification of a particular strain and ready comparison of results between different laboratories (48). MLST has provided valuable insights into the national and international epidemiology of MRSA but lacks the discriminatory power for investigating local epidemiology when there is a high prevalence of epidemic strains such as EMRSA-15 or -16. Staphylococcal Protein A (spa) typing requires the sequencing of a short sequence repeat (SSR) region in *spa*, and has a greater discriminatory power than MLST (50).

## 1.8 Project hypothesis and aims of the study

The purpose of this study was to determine the MRSA profiles for isolates collected from three Arab countries, against a panel of 14 antibiotics and place this information within the context of current prescribing policies in these countries. In addition, the study will determine the heterogeneity of the *spa* gene by sequencing. This approach would be able to show a possible clonal variability in these MRSA strains that was not demonstrated in previously published works. The specific aims were:

1. To determine the resistance pattern of MRSA against a panel of 14 antibiotics.

2. To detect *mecA* gene by Hybridization based PCR (Genotype MRSA

Direct test) from MRSA identified by oxacillin disc diffusion.

3. To analyze the clonality of the most common MRSA strains encountered in Palestine, Jordan and Iraq, and to analyze molecular characteristics of isolates in order to identify those linked to epidemics

# Chapter Two

# Materials and methods

## 2.1 Collection of *S. aureus* isolates

Sixty seven clinical MRSA isolates from three different Arab countries were included in this study. Seventeen isolates were from West Bank Palestine, 25 isolates from Jordan, and 25 isolates from Iraq. MRSA were recovered from urine, wound, sputum, nasal, skin and body fluids.

## 2.2 Identification of S .aureus

Isolates were identified as *S. aureus* according to colony and microscopic morphology, positive catalase, and coagulase production.

## 2.3 MRSA Determination Oxacillin susceptibility

Screening for methicillin resistant S. *aureus* isolates were detected using the disk diffusion method outlined by the National Committee for Clinical Laboratory Standards (NCCLS) (51). One µg oxacillin disks were placed on Muller-Hinton agar (oxoid, United Kingdom) supplemented with 4 % NaCl. Inoculum size was adjusted to a final concentration of $1\times10^5$ cfu/ml. The Zones diameter was measured following incubation at 35°C for 24 hours. Isolates with a zone diameter ≤10 mm were considered methicillin resistant.

## 2.4 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed by disc diffusion as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (51). All antibiotic discs were obtained from oxoid The following antimicrobial agents were tested: oxacillin (1µg), penicillinG (1µg), erythromycin (15µg), clindamycin (2µg), vancomycin (30µg), ciprofloxacin (5µg), gentamicin (10µg), cefotaxime (30µg), fusidic acid(10µg), amikacin(30µg), teicoplanin (30µg), imipenem (10µg) ceftazidime (30µg) and sulfamethoxazole-trimethoprim (1.25/23.75 µg). *Staphylococcus aureus* ATCC 25923 was used for the quality control of the antibiotic susceptibility procedure. The test was carried out as follows: One colony from an overnight culture (18- 24 hours) was inoculated into 0.85% saline solution. suspension was adjusted to 0.5 McFarland standard. A sterile cotton swab was dipped into the culture and a bacterial lawn was made on a Mueller- Hinton (MH) agar plate. The plate was allowed to stand for 3 to 15 minutes before placing the antibiotic discs. Antibiotic discs were aseptically placed on the surface of the M-H agar plats using a sterile forceps. The Plates were incubated at 35°C within 15 minutes of disc diffusion. for 18 to 24 hours. The diameter of the zone of inhibition was accurately measured and as sensitive, intermediate or resistant (SIR) according to the recommendations of the National Committee for Clinical Laboratory Standards(51).

## 2.5 Geno Type MRSA test

All MRSA isolates determined by the susceptibility to oxacillin were confirmed by PCR for the mecA gene (GenoType MRSA Direct; Hain Lifescience, Nehren, Germany). Briefly, the test assay consists of a three-step protocol: (i) isolation of DNA from cultured material, (ii) multiplex amplification with biotinylated primers, (iii) and reverse hybridization. The reverse hybridization included the following steps: chemical denaturation of the amplified products, hybridization of the single-stranded biotin-labeled amplicon to membrane-bound probes, stringent washing, addition of a streptavidin-alkaline phosphatase conjugate, and a staining reaction. All steps were performed according to the manufacturer's instructions.

### 2.5.1 DNA Extraction

One Bacterial colony growing on blood agar plates, (was taken using 1µ loop), was resuspended in 50 µl of lysostaphin (100 µg/ml in water; Sigma chemical Co, USA) in micro centrifuge tubes. The Cell suspensions were incubated at 37°C, for 10 min, followed by the addition of 50 µl proteinase K solution (100 µg/ml, Sigma, USA) and 150 µl of Tris buffer (0.1 M Tris, PH 7.5). Cell suspensions were incubated for an additional 10 min at 37°C and then placed in a boiling water bath for 5 minutes. The tubes were then centrifuged at 4500 rpm for 2minute to precipitate the DNA.

Extraction and purification of genomic DNA in the pellet was carried out using QIAamp DNA mini and blood kit according to the manufacturer's instructions (Qiagen, USA). One percent agarose gel was used to check the quality of extracted DNA. The purity of DNA was checked optically at 260nm and 280nm.

### 2.5.2 Multiplex amplification with biotinylated primers

The Genotype MRSA assay was performed by PCR as recommended by the manufacturer. The primers sequences used and conditions are summarized in Table 2.1 Briefly, 35 µl of a primer nucleotide mixture (provided with the kit), was placed in a PCR tube, amplification buffer containing 15 mM $MgCl_2$ and 1 U of Hot StarTaq polymerase (Qiagen, USA), and 5 µl of DNA in a total volume of 50 µl was then added. Each run included a pure water control to demonstrate the absence of contaminating DNA. The sensitivity of amplification and hybridization was monitored using an internal control.

### 2.5.3 Hybridization

Hybridization and detection were carried out with an automated washing and shaking device. Twenty microliters of the amplification products was mixed with 20 µl of denaturing reagent (provided with the kit) for 5 min. This was followed by addition of 1 ml of prewarmed hybridization buffer, and membrane strips were placed in each trough. Hybridization took place at 45°Cfor 30 min, followed by two washing

steps. For colorimetric detection of hybridized amplicons, streptavidin-conjugated alkaline phosphatase and the appropriate substrate were added. After final washing, the strips were air dried and fixed on paper.

**Table 2.1 Primer sets used in polymerase chain reaction PCR-based**

| Target genes | Primer sequence (5`-3`) | Annealing temperature (°C) |
|---|---|---|
| **16 S ribosomal DNA F**<br>**16 S ribosomal DNA R** | 5'-AGG CCC GGG AAC GTA TTC AC-3'<br>5'-GAG GAA GGT GGG GAT GAC GT-3' | 60 |
| ***S aureus* species F**<br>***S aureus* species R** | 5'-GTT GTA GTT GTC GGG TTT GG-3'<br>5'CTT CCA CAT ACC ATC TTC TTT AAC-3' | 60 |
| ***S epidermidis* F**<br>***S epidermidis* R** | 5'-GTC GGT ACA CGA TAT TCT TCA CG-3'<br>5'-CTC TCG TAT GAC CAG CTT CGG TAC-3' | 60 |
| **mecA – F**<br>**mecA – R** | 5'-CAA GAT ATG AAG TGG TAA ATG GT-3'<br>5'-TTT ACG ACT TGT TGC ATA CCA TC-3' | 60 |
| **PVL F**<br>**PVL R** | 5’-CAA GAT ATG AAG TGG TAA ATG GT3’<br>5’TTT ACG ACT TGT TGC ATA CCA TC3’ | 60 |

### 2.5.4 Interpretation:

The strips were examined for the presence of purple colored lines indicating positive hybridization. All individual steps (DNA isolation, DNA amplification, and hybridization) are monitored by a universal internal control to ensure the reliability of the test. This internal control uses primers and a probe targeting a conservative part of the bacterial genome. When faint bands appears, the intensity of the universal control band was used to evaluate the strip. Bands with color intensity equal to or greater than that of the control band were considered positive. Analytical sensitivity and specificity were calculated with defined reference strains such as *S. epidermidis*.

## 2.6 spa sequencing

### 2.6.1 DNA Amplification

For amplification of the *Staphylococcus* protein A *(spa)* repeat region, PCR was performed in a total volume of 50 µl containing cleaned DNA eluted from the membranes, 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP,and dTTP), 10 pmol of each primer, 5 µl of 10-fold concentrated PCR Buffer MgCl2 1.5 mM, and 1.25 U of AmpliTaq DNA polymerase. Thermal cycling reactions consisted of an initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation of 45 s at 94°C, annealing of 45 s at 60°C, and extension of 90 s at 72°C, with a single final extension of 10 min at 72°C. HPLC-cleaned primers spa-1113f (5'-TAA AGA CGA TCC TTC GGT GAG C-3') and spa-1514r (5'-CAGCAG TAG TGC CGT TTG CTT-3') were used for amplification.

### 2.6.2 PCR Purification

This step was essential to eliminate undesirable components that may be present and to obtain pure concentrated product adequate for sequencing. Purification of the positive PCR products was conducted by using the MinElutePCR purification kit (Qiagen, Germany). The purified PCR products were then re-amplified in a total volume of 50µl. as shown previously. Purification of the PCR product was carried out before sequencing as follows: 250 µl of sodium iodide was added to 50 µl of the

positive PCR product.

The mixture was then gently vortexed and added to the DNA binding column, centrifuged to elute unbound components and washed twice with the washing buffer included in the kit. Bound DNA (PCR product) was eluted with 10 µl $ddH_2O$.

Agarose gel electrophoresis was then carried out using 2 µl products to ensure purity and concentration prior to sequencing.

**2.6.3 DNA sequencing**

Sequencing reactions were carried out by a dideoxy chain termination method using an ABI PRISM Model 301 Sequence Instrument at Bethlehem University, Bethlehem, Palestine.

**2.6.4 DNA analysis.**

DNA analysis and sequence comparisons were conducted using CLC Main Workbench software; version 5.6.1, 2009). The phylogenetic tree was constructed by employing the neighbour-joining algorithm in the program NEIGHBOR in the CLCL program package version 5.6.1.

Spa types were designated using spa typer of Fortinbras Research (http://fortinbras.us/ht/FRblog.html).

**2.7 Statistical analyses**

Statistical analysis was performed using the program Statistical Package for Social Sciences (SPSS, Chicago, IL, USA). The significant differences in resistance rates was determined by means of the Z-test. A statistically significant difference was defined as a p value of $< 0.05$ and 95% confidence interval.

# Chapter Three

# Results

## 3.1 Detection of MRSA by the Geno Type MRSA Direct

The present study evaluated a PCR-based hybridization assay (GenoType MRSA Direct) for the direct detection of MRSA. This test is based on amplification of both a part of the *mecA* gene and a sequence specific for *Staphylococcus aureus*, followed by subsequent hybridization of the denatured amplicons to their complementary sequences coated to membrane strips. The hybridization of amplicons is detected by the streptavidin-alkaline phosphatase complex, with a purple-colored line indicating a positive result (52-54).

Hybridization examples of the Geno Type MRSA results of six of the MRSA isolates are shown in Figure 3.1. The relationship between the results of the Geno Type MRSA assay and the disk diffusion test results for oxacillin are summarized in Table 3.1. As can be deduced from Table 3.1, all the sixty seven strains (100%), which appeared methicillin resistant phenotypically with antimicrobial susceptibility testing were positive for Geno Type MRSA assay specific for the *mec*A gene. Consequently, the sensitivity and specificity of Geno Type MRSA assay as compared to oxacillin disk diffusion test are therefore 100%.

SCC*mec* type IV or V which is associated with (PVL)phenotypes was detected in 3% of MRSA isolates. These MRSA phenotypes were isolated from Iraq, but none were isolated from Palestine nor Jordan (Table 3.1).

## 3.2 Antimicrobial susceptibility testing

The results of antimicrobial susceptibility testing reflect the scope and seriousness of the problem of methicillin resistance among the *S. aureus* isolates in these three neighboring countries (Palestine, Jordan and Iraq). As shown in Table 3.2, the MRSA isolates were predominantly resistant to a greater range of antibiotics. Of the fourteen antibiotics tested, isolates indicated extremely high rate of resistance (above 82%) to oxacillin, penicillinG, erythromycin, clindamycin, ciprofloxacin, gentamicin, cefotaxime, fusidic acid, ceftazidime and sulfamethoxazole-trimethoprim. Resistance rates of MRSA to other antibiotics were 64.2% to each of amikacin and imipenem, 13.4% to teicoplanin. None of the 67 isolates were resistant to vancomycin.

To detect the presence of significant difference among antibiotic resistance between countries, a Z-test was conducted. The results revealed that the rate of resistance among MRSA isolates to cefotaxime was higher among Palestinian than Joradanian or Iraqi isolates (Table 3.2) and this difference was statistically significant ($p < 0.05$).

Multidrug resistance (MDR) is defined as resistance to three or more different classes of antibiotics . In this study MDR was detected in 45 isolates (67.2%). Our results showed that these isolates were resistant to at least five classes of antibiotics: penicillins (oxacillin, penicillin), macrolids (erythromycin), incosamides (clindamycin), aminoglycosides (gentamicin) and fluoroquinoleres (ciprofloxacin). Statistical analysis showed a significant difference ($p < 0.05$) in Palestinian MDR-MRSA isolates that are resistant to nine or more antibiotics as compared to Jordanian or Iraqi isolates (Figure 3.2).

Although the Palestine isolate group showed higher rates of resistance to gentamicin, amikacin, imipenem, sulfamethoxazole-trimethoprim and cefotaxime as compared to Jordan or Iraq isolate groups, There was no significant difference detected by SPSS to teicoplanin (Table 3.2).

As shown in Table 3.3, there were two different antibiotic resistance patterns indentified within the three MRSA groups. (Jordan , Iraq ,Palestine ) pattern.

Pattern number one was the predominant . Resistance rates encountered were 41% (7/17) in Palestine , 36% (9/25) in Jordan and 28% (7/25) in Iraq. Less predominant was pattern number three as shown in Table 3.3. The remaining results among the rest of profiles does not

seen to be significant and therefore neglected . Representative samples were closen randomly from groups one and three and sequenced .

## 3.3 Phylogenetic analysis

Nucleotide comparison and maximum likelihood phylogenetic analysis created by a Clustal tree using version 5.6.1 of CLCL Main Workbench of 11 representative MRSA isolate from the predominant antibiotic resistance patterns (1 and 3) of the three study groups and other Gen Bank-accessible gene sequences of MRSA are shown in Figure 3.2 and Figure (3.4.) The results indicate the existence of two main clusters of MRSA in these three countries. Cluster (I) comprised 18.2% (n=2) of the isolates, and contained one representative isolates from each of Jordan and Iraq. The second cluster (II) comprised most of the strains 81.8% (n=9), and contained 5 representative isolates from Palestine, and two isolate from each of Jordan and Iraq. The tree generated in Figure 3.4 also shows that isolates of cluster II are the closest relative to strain with an accession number X61307, AM407300, U54636 and EF094528 which belong to France, Switzerland, Brazil and Australia, respectively.

Three spa types were identified in the 11 tested isolates. These types include t386 and t037 Table 3.4, whereas the other 8 isolates do not exist in the database of the used software since the database of this

program is not updated since 2009. Accordingly, we don't include these data in our study.

**Table 3.1 Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) by the Geno Type MRSA Direct test in comparison with methicillin disk diffusion test.**

| Country of Origin (MRSA) | No. of isolates | Oxacillin | | Result of Geno Type MRSA | | | |
|---|---|---|---|---|---|---|---|
| | | | | *mecA (n)* | | PVL | |
| | | Pos. | Neg. | Pos. | Neg. | Pos. | Neg. |
| Palestine | 17 | 17 | None | 17 | None | | 17 |
| Jordan | 25 | 25 | None | 25 | None | | 25 |
| Iraq | 25 | 25 | None | 25 | None | 2 | 23 |

**Table 3.2 Resistance of MRSA isolates to various antibiotics each hospital from the Palestine, Jordan and Iraq.**

| | AMK | CAZ | CIP | CLI | CTX | ERY | FUS | GEN | IMP | SXT | TPL | VAN | OXA | PEN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Palestine** | **76.5** | **100** | **94.1** | **100** | **100** | **94.1** | **100** | **94.1** | **70.6** | **94.1** | **11.8** | **0** | **100** | **100** |
| **Jordan** | **60.0** | **100** | **100** | **100** | **76.0** | **96.0** | **100** | **80.0** | **64.0** | **80.0** | **16.0** | **0** | **100** | **100** |
| **Iraq** | **60.0** | **100** | **92.0** | **100** | **76.0** | **96.0** | **100** | **76.0** | **60.0** | **76.0** | **12.0** | **0** | **100** | **100** |
| **Total** | **64.2** | **100** | **95.5** | **100** | **82.1** | **95.5** | **100** | **82.1** | **64.2** | **82.1** | **13.4** | **0** | **100** | **100** |

AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CLI, clindamycin; CTX, cefotaxime; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; IMP, imipenem; SXT, sulfamethoxazole-trimethoprim; TPL, teicoplanin; VAN, vancomycin; OXA, oxacillin; PEN, penicillin.

**Table 3.3 Patterns of resistance to individual antibiotics among the MRSA isolates from the Palestine, Jordan and Iraq.**

| Resistance pattern | | | | | | | | | | | | | | (%). of isolates showing the indicated resistance pattern. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | Palestine (n=17) | Jordan (n=25) | Iraq (n=25) |
| AMK | CAZ | CIP | CLI | CTX | ERY | FUS | GEN | IMP | SXT | TPL | VAN | OXA | PEN | | | |
| R | R | R | R | R | R | R | R | R | R | S | S | R | R | 41 | 36 | 28 |
| S | R | R | R | S | R | R | S | S | S | S | S | R | R | 00 | 08 | 16 |
| R | R | R | R | R | R | R | R | R | R | R | S | R | R | 12 | 16 | 12 |
| R | R | R | R | R | R | R | R | S | R | S | S | R | R | 12 | 04 | 08 |
| R | R | S | R | R | R | R | R | R | R | S | S | R | R | 06 | 00 | 08 |
| S | R | R | R | R | R | R | R | R | R | S | S | R | R | 06 | 08 | 08 |
| S | R | R | R | R | R | R | R | S | R | S | S | R | R | 11 | 04 | 04 |
| S | R | R | R | R | R | R | S | R | R | S | S | R | R | 06 | 04 | 04 |
| R | R | R | R | R | S | R | R | S | S | S | S | R | R | 06 | 04 | 04 |
| S | R | R | R | S | R | R | S | S | R | S | S | R | R | 00 | 08 | 04 |
| S | R | R | R | S | R | R | R | S | S | S | S | R | R | 00 | 08 | 04 |

AMK, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CLI, clindamycin; CTX, cefotaxime; ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; IMP, imipenem; SXT, sulfamethoxazole-trimethoprim; TPL, teicoplanin; VAN, vancomycin; OXA, oxacillin; PEN, penicillin.

**Table 3.4 Spa typing of 11 selected MRSA isolates.**

| sequence | [1]Start | [2]Repeat units | [3]Len in bp | [4]Repeat seq | [5]ridom type |
|---|---|---|---|---|---|
| 2-F_P_1 | 43 | 3 | 3 | U1:J1:E1 | t386 |
| 8-F_P_1 | 44 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 13-F_P_1 | 49 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 27-F_P_1 | 47 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 28-F_P_1 | 45 | 7 | 168 | W1:*:K1:A1:O1:M1:Q1 15:*:16:02:25:17:24 | * |
| 31-F_J_1 | 45 | 6 | 168 | W1:G1:K1:A1:O1:M1:Q1 15:12:16:02:25:17:24 | t037 |
| 41-F_J_1 | 47 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 37-F_J_1 | 50 | 7 | 167 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 25-F_I_1 | 55 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 47-F_I_1 | 51 | 7 | 168 | *:G1:K1:A1:O1:M1:Q1 *:12:16:02:25:17:24 | * |
| 48-F_I_1 | 68 | 6 | 144 | G1:K1:A1:O1:M1:Q1 12:16:02:25:17:24 | t386 |

[1]Starting coordinate of repeats in sequence; [2]Number of repeat units; [3]Length of entire VNTR; [4]Kreiswirth, Ridom nomenclature; [5]***** indicates a sequence that is a likely spa repeat, but does not exist in the database used of the program.

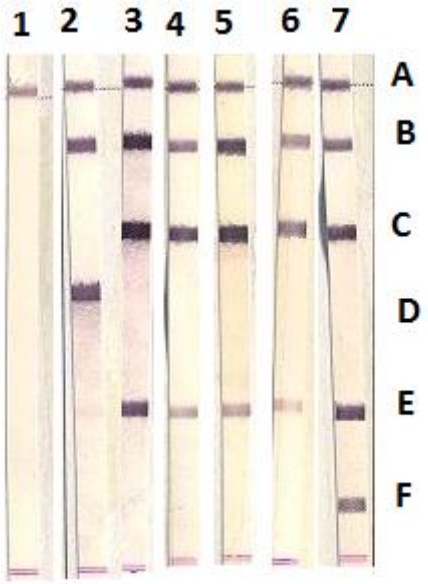


**Figure 3.1** Representative hybridization of MRSA isolates with the GenoType MRSA Direct. Lane 1, water control; 2, *S. epidermidis*; 3-6, MRSA. 7, MRSA positive PVL gene A, conjugate control; B, universal control; C, S. aureus; D, *S. epidermidis*; E, *mec* A gene; F, PVL gene.

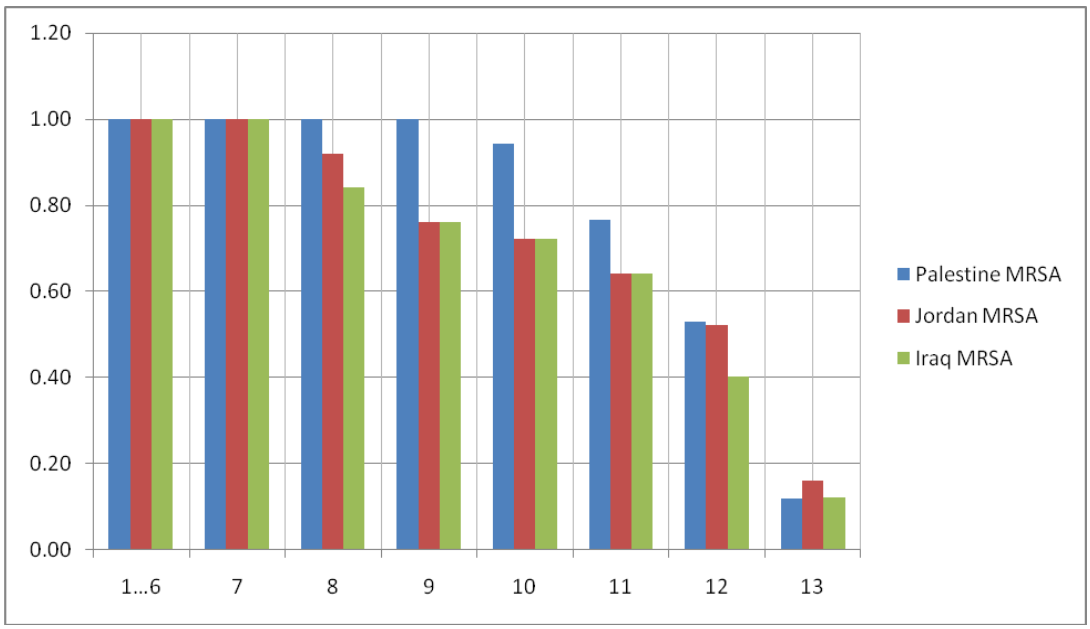


**Figure 3.2** The percentage of isolates within a specified study group showing resistance to a given number of antibiotics. X axis, number of antibiotics; Y axis, The percentage of isolates showing resistance to a given number of antibiotics.

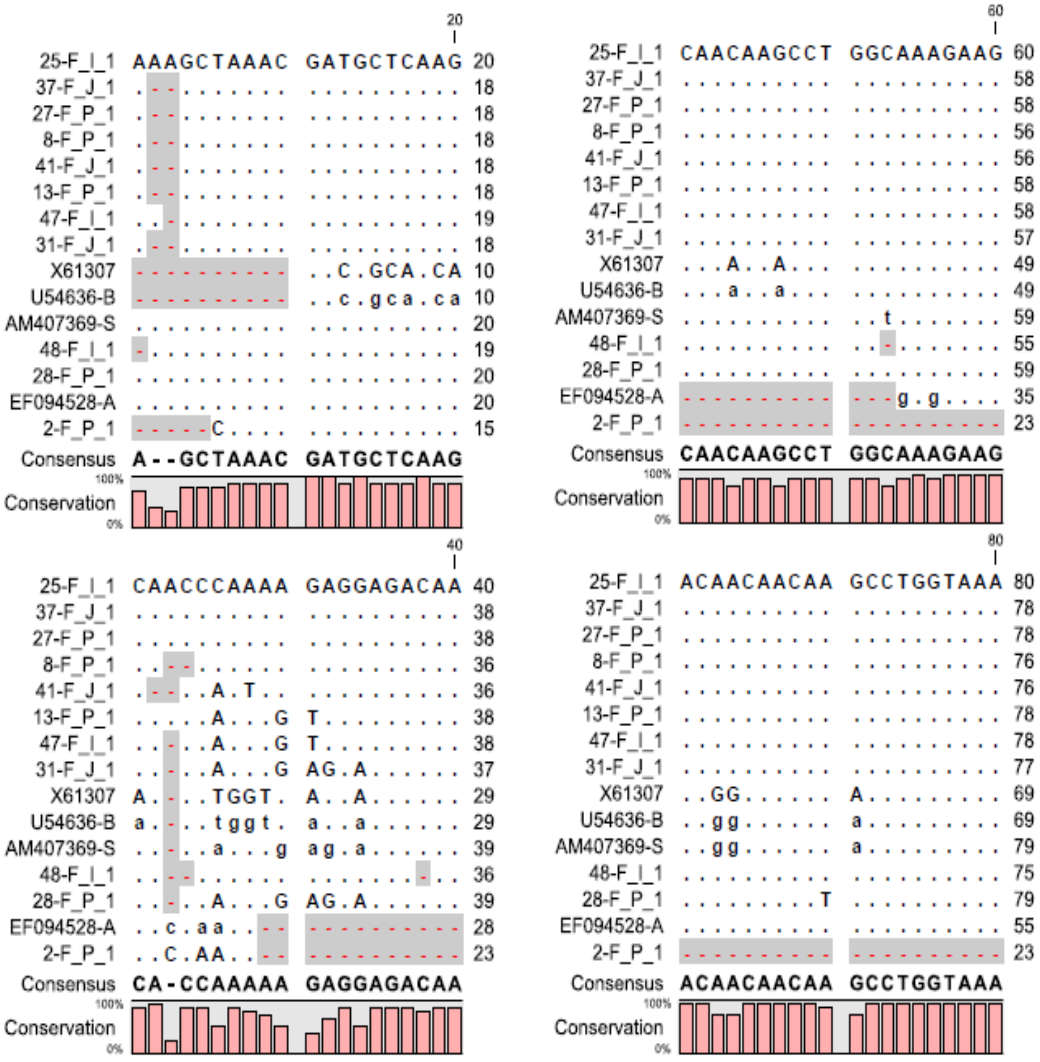


**Figure 3.3** Nucleotide comparison between *spa* gene of 11 representative MRSA isolates and other Gen Bank-accessible *spa* gene sequences of MRSA strains. Dots represent nucleotides similar to the consensus.

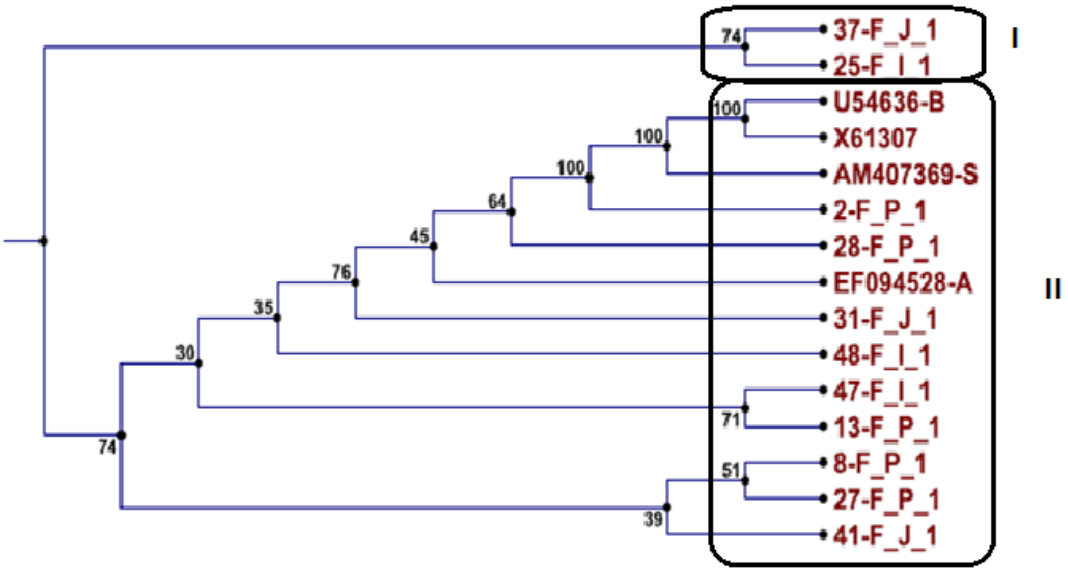


**Figure 3.4** A phylogenetic tree based on the partial nucleotide sequences of the *spa* gene of 11 selected MRSA isolates and Gen Bank-accessible *spa* gene sequences of MRSA. The tree is rooted to version 5.6.1 of CLCL Main Workbench.

# Chapter Four

## Discussion

MRSA is now spreed in many areas of the world. The frequency of infections and outbreaks due to MRSA have continued to increase. MRSA is often multidrug resistant and therapeutic options are limited causing increased concern (55-57).

One important approach for controlling MRSA transmission in hospitals involves the rapid identification of MRSA- Carriers among patients as well as health care workers.

Delays in detection of MRSA can lead to increased transmission among patients, high frequency of infections, and increased hospital costs. Therefore one of the objectives of this study was to evaluate and implement a rabid, highly specific and sensitive method for MRSA detection, GenoType MRSA Direct. This Method contains primers specific for staphylococcal cassette chromosome *mec* (SCC*mec*).

The use of GenoType MRSA Direct for detection of *S. aureus* species specific 16S rRNA. SCC*mec*A and PVL genes, is simultaneous and a rapid method. The involvement of PVL gene with severe skin and soft tissue infections, especially necrotizing skin infections is considered a stable marker for CA-MRSA (58).

In addition to its high specificity and sensitivity, the results of the test are obtained within four hours.

As shown in Table (3.1), there is consensus (100%) between phenotypic characterization of MRSA by antimicrobial susceptibility test and GenoType MRSA assay specific for the *mec*A gene. Considering the high sensitivity and specificity of GenoType MRSA assay as compared to methicillin disk diffusion test the GenoType assay can be used as the test of choice and to confirm the identification of S.*aureus* and detection of MRSA.

In conclusion, it is apparent that the GenoType MRSA Direct assay is rapid, sensitive and specific. The value of adopting this assay in major hospitals and clinics reflects on improving the efficiency and effectiveness of infection control measures to prevent the spread of MRSA.

Our results conform with those repeated by Huletsky et al. (59) who adopted a similar system to monitor MRSA for hospital surveillance program.

MRSA strains harboring the PVL genes and SCCmecA was detected in only 3% of the isolates (2/67) , characterizing them as CA-MRSA

These MRSA strains were isolated from Iraq, but not from Palestine or Jordan (Table 1).

The prevalence of CA-MSRA reported in neighboring Arab countries is similar to the results obtained in this work (24). CA-MRSA remains relatively low compared to those reported in many other countries (60-62).

In the present study, we evaluated the antimicrobial susceptibility of all MRSA strains against a panel of fourteen antibiotics. The results indicated that methicillin resistance has become a serious problem in these countries. Although the reduced number of tested samples from these groups limits generalizations, an extremely high rate (88%) of resistance to oxacillin, penicillin G, erythromycin, clindamycin, ciprofloxacin, gentamicin, cefotaxime, fusidic acid, amikacin, teicoplanin, imipenem, ceftazidime and sulfamethoxazole-trimethoprim was observed, similar findings were reported in several countries (55-57, 63).

Multidrug resistance was observed in 40% of the isolates. While all the isolates were susceptible to vancomycin, resistance was seen in 13.43% (9/67) to teicoplanin.

This was not surprising since these antibiotics are not routinely used to treat infections caused by S.*aureus* (no-selective pressure) to these antibiotics is currently rare.

Beside oxacillin, (above 80%) of the MRSA isolates encountered in this study were resistant to β-lactams, The extreme resistance to the β-

lactams is unsurprising as all isolates in Figure 2 are MRSA and therefore inherently resistant to this class of antibiotic. Resistance seen to erythromycin and clindamycin, may in part be due to a single resistance mechanism that affect these two antibiotics. Erythromycin and clindamycin share a similar mode of action against the bacterial cell effecting the 50s ribosomal subunit and belong to the macrolides. Two mechanisms of macrolide resistance have been described. First, target modification mediated by an rRNA *erm* gene methylase that alters a site in 23S rRNA common to binding of macrolides, whereas the second mechanisms of resistance is mediated by two classes of active efflux pumps (64).

In our study, the vast majority of MRSA isolates showed cross-resistance to all classes of antibiotics used, leaving few therapeutic options to treat infections caused by this microorganism. This phenomenon may be related to the dissemination of transposons with insertion sequences in the 50-kb *mec* region gene (65-66). For example, the ability of IS*431* elements through homologous recombination to trap and cluster resistance determinants with similar IS elements explains the multiple drug resistance phenotype that is characteristic of MRSA strains (67-68).

The rates of resistance to cefotaxime was higher among Palestinian MRSA isolates than Joradanian and Iraqi isolates (Table 3.2) and this

difference was statistically significant ($p<0.05$). Likewise, multidrug resistance occurred significantly ($p<0.05$) among Palestinian isolates more frequently than Jordanian and Iraqi isolates, namely to gentamicin, amikacin, imipenem, sulfamethoxazole-trimethoprim and cefotaxime. This high rate is probably due, in part, to the selective pressure resulting from uncontrolled, prescription of gentamicin , imeprnem, penicillin and its derivatives to treat MSSA infection. Thus, the use of these antibiotic would be limited, due to high levels of resistance (60%).

The MRSA isolates from the three countries were resistant to between 2 and 10 antibiotics, with a mean resistance to six of the antimicrobials in the panel (Table 3.3). These observations placed additional urgency to understand the mechanisms of resistance to methicillin as well as second line antibiotics. For isolates that are methicillin-resistant, vancomycin is advocated (68). MDR profiles as well as resistance levels to individual antibiotics were determined from the data in this study. The predominant MDR profiles can be seen in Table 3.3. It contained a combination of twelve antibiotics. These antibiotics are oxacillin, penicillin, erythromycin, clindamycin, ciprofloxacin, gentamicin, cefotaxime, fusidic acid, amikacin, imipenem, ceftazidime, sulfamethoxazole-trimethoprim. These data brought up several items which call for more precise protocols to treat infections caused by this dreadful organism.

A recent review has highlighted the need for better antibiotic stewardship in the possible form of “antibiotic care bundle” (65-66). The primary goal of this protocol is to provide a practical framework for promoting and reinforcing optimal prescribing behaviour, to ensure that all patients receive the right antibiotic therapy, at the right dose, route and duration, for the right bacterial infection at the right time. In addititon, antimicrobial cycling **“**the scheduled substitution of a specific antimicrobial agent or class for another agent or class to prevent or reverse antimicrobial resistance” provides heterogeneity in antimicrobial use, may minimize the selection pressure that leads to resistance (69).

Nucleotide comparison and phylogenetic analysis provides a method of assigning strains to groups on the basis of similarities. As can be deduced from Figure 3.3 and 3.4, cluster analysis revealed one large cluster containing strains isolated from the three studied countries and a second small cluster containing only strains isolated in Jordan and Iraq. Inside these clusters there were strains that showed 100% similarity to others strains. Independent of the strain numbers isolated in each area, it is possible to observe that strains from one geographical area tend to evolve forming predominant successful clones responding to specific selective pressures and strains from one geographical area can present genetic proximity with strains from another geographical area. These findings support the data published by Montesinos et al. (70).

The genetic profiles as well as resistance profile to antibiotics observed show genetic spread events through our MRSA population. The possible existence of two main MRSA clusters in the three countries might be explained to local selective pressure resulting from uncontrolled, inappropriate and frequent administration of broad-spectrum antibiotics to treat MRSA infections. This selective pressure force horizontal genetic recombinant events. Although recombination in *S. aureus* is uncommon, our data, indicating that MRSA has a genetic diversity, even if we are analyzing gene that should not diverge (spa gene) as mentioned elsewhere (71-72).

Palestine, Jordan and Iraq are Arab countries which share the geographical boundaries. Cross-border patient mobility within these countries in general, between Palestine and Jordan in particular, and between Iraq and Jordan during and after the occupation of Iraqi in 2003 is an important issue of concern. In this regard, it is interesting to note that these countries share *spa* and other resistance genes. Consequently, the cross-border transfer of patients may have an important impact on the dissemination and prevalence of MRSA in particular clone II. An interesting issue in the phylogenetic analysis of this study is the relationship between isolates of cluster II and the international strains with an accession number X61307, AM407300, U54636 and EF094528 which belong to France, Switzerland, Brazil and Australia, respectively.

Taken together, these results suggest that two epidemiologically distinct MRSA clusters exist in the three countries and cluster II is global clonally disseminated, often multiresistant strains, which poses a global imminent threat. Thus further studies are needed for the surveillance of MRSA strains using a combination of molecular techniques (SCC*mec* typing, PFGE, MLST) typing, to relate our strains to international epidemic clones and to provide a much larger view of molecular epidemiology of MRSA and isolates in Arab world.

## Conclusion and Recommendations

1. MRSA has clearly emerged as a serious problem in Palestine, Jordan and Iraq.

2. Antimicrobial resistance has clearly emerged as a serious problem with MRSA in Palestine, Jordan and Iraq. By analogy on the basis of experiences in other parts of the world, this problem is likely to grow in the future. Thus, more aggressive microbiological and infection control policies are necessary to prevent the further spread of these microorganisms.

3. The GenoType MRSA Direct assay appears to be a rapid, sensitive and specific test for direct detection of MRSA. The assay provides same day

results, thereby improving the efficiency and effectiveness of infection control measures to prevent the spread of MRSA in a hospital setting.

4. Nucleotide alignment and Phylogenetic tree analysis of *spa* gene revealed that the current representative isolates of MRSA were genetically close to global isolates.

5. The presence of MRSA cluster spread should provide important motivation to reinforce routine infection control procedures to limit horizontal transmission of MRSA.

**Base on the outcome results, the following recommendations should be made to reduce the risk of MRSA infection in the Arab world:**

1. Continued monitoring of local antibiotic resistance profiles in MRSA, allowing for the production of responsive therapy regimens in response to ever changing microbial resistance profiles

2. A detailed understanding of the molecular/genetic basis, evolution and disemmination of resistance to antibiotics in MRSA.

3. The needed for the surveillance of MRSA strains using a combination of molecular typing techniques, to relate our strains to international epidemic clones and to provide a much larger view of molecular epidemiology of MRSA and isolates in Arab world.

4. To establish *spa* typing software in our molecular laboratory. This program can be used in the future for MRSA surveillance and comparison

of our strains with international epidemic clones. It ensures a common typing nomenclature and thus greatly facilitates the exchange of typing data for *S. aureus*. In the future, this typing method may become as a gold standard typing method, since this sequence-based method is much more affordable and quicker to perform.

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