An-Najah National University
Faculty of Graduate Studies

Application of Antibiogram, Ribosome Spacer PCR (RS-PCR), and Arbitrarily Primed PCR (AP-PCR) for Typing of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

By

Ahmad Mahmoud Hasan Saleh

Under the Supervision

of

Dr. Kamel Adwan, Dr. Nael Abu-Hasan

and Dr. Tamer Essawi

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biological Sciences, Faculty of Graduate Studies

An-Najah National University

Nablus, Palestine.

May, 2001

Application of Antibiogram, Ribosome Spacer PCR (RS-PCR), and Arbitrarily Primed PCR (AP-PCR) for Typing of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

By

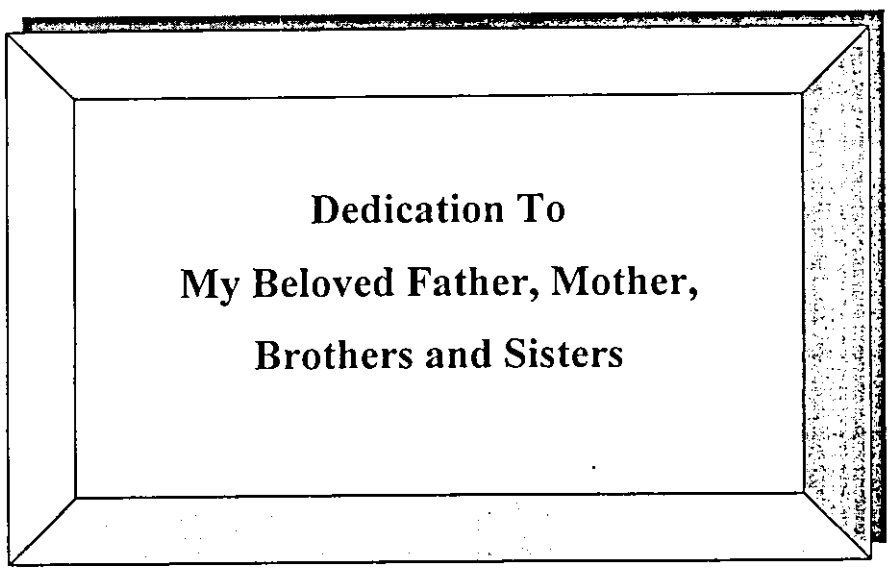
Ahmad Mahmoud Hasan Saleh

Date of Defence : May 23, 2001

This thesis was defended successfully on

and approved by

Committee Members		Signature
1. Dr. Kamel Adwan	(Advisor)	
2. Dr. Nael S. Abu - Hasan	(Advisor)	
3. Dr. Tamer Essawi	(Advisor)	
4. Dr. Yahia Faydi	(Internal Examiner)	
5. Dr. Gabi Abu Sa'da	(External Examiner)	



ACKNOWLEDGEMENTS

I would like to express my profound gratitude to Dr. Kamel Adwan, Dr. Nael Abu-Hasan and Dr. Tamer Essawi for their valuable assistance and direct supervision through the course of this work and during the course of writing up this thesis.

Special thanks for my family and faithful relatives for their encouragement and support.

My deep thanks to my colleagues in the Islamiya Secondary School for their encouragement and kind cooperation during this work.

My thanks also to Ala' Abu Zant, Marwan Budair, and other colleagues for their help and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

RS-PCR: Ribosome Spacer Polymerase Chain Reaction

AP-PCR: Arbitrarily Primed Polymerase Chain Reaction

MRSA: Methicillin Resistant *Staphylococcus aureus*

EMRSA: Epidemic Methicillin Resistant *Staphylococcus aureus*

SMRSA: Sporadic Methicillin Resistant *Staphylococcus aureus*

rDNA: ribosomal Deoxyribo Nucleic Acid

PBPs: Penicillin Binding Proteins

PDG: Peptidoglycan

ATP: Adenosine Tri Phosphate

CNISP: Canadian Nosocomial Infection Surveillance Program

PFGE: Pulsed Field Gel Electrophoresis

HVR: Hypervariable Region

RFLP: Restriction Fragment Length Polymorphism

MLEE: Multilocus Enzyme Electrophoresis

CFU: Colony Forming Unit

MIC : Minimal Inhibitory Concentration

NCCLS: National Committee for Clinical Laboratory Standards

EDTA: Ethylene Diamine Tetra acetic Acid

TBE: Tris Borate EDTA

CS: Coefficient of Similarity

NU: Neonatal Unit

ICU: Intensive Care Unit

HCW: Health Care Worker

ABSTRACT

Nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) represent an increasing problem in hospitals worldwide. Quick and reliable typing methods are required to obtain information about the relatedness of MRSA isolates and to allow faster implementation of appropriate infection control measures.

The current study describes the distribution of forty four MRSA isolates collected between February and May 1998 from 3 hospitals in Palestine, and the ability of antibiogram and two different genotyping techniques - Ribosome Spacer PCR (RS-PCR) and Arbitrarily Primed PCR (AP-PCR), to detect the relatedness among the MRSA isolates.

The ability of these techniques to detect different types among the MRSA isolates was found to be as follows; seventeen antibiogram types were noted by using 15 different antimicrobial agents, designated 1 through 17. MRSA isolates were resistant to the majority of antimicrobial agents tested; oxacillin (100%), ampicillin (100%), clindamycin (95.5%), erythromycin (86.4%), tetracyclin (72.7%), and trimethoprime (61.4%). However, all isolates were susceptible to both vancomycin and refampicin. Around 78% of the isolates were found to be resistant to more than 3 different antimicrobial agents. RS-PCR generated eleven different genotypes among the 40 examined isolates and 4 of the isolates were non-typeable, designated I through XI; each spacer pattern was represented by 1-6 fragments ranging from 300-800bp. AP-PCR identified fifteen different genotypes among the 38 MRSA examined and 6 isolates were non typeable using the BG2 arbitrarily primer (GGT TGG GTG AGA ATT GCA CG; 5' to 3'), designated A through O; each BG2 pattern was represented by 1-7 fragments ranging from 300-800 bp.

AP-PCR using P7 arbitrarily primer (GTG GAT GCG A; 5' to 3') revealed fourteen distinct genotypes among the 42 MRSA isolates and two isolates were non-typeable, designated a through n. The revealed fragments size (1-11) ranged from 300-900 bp.

In our study, genotyping was more powerful tool than antibiogram in differentiating between unrelated isolates of MRSA, and strongly indicates that the source of MRSA was the environment in both the neonatal unit (NU) and intesive care unit (ICU), since most of the environmental and colonized MRSA isolates of both units revealed identical genetic patterns.

Combination of RS-PCR and AP-PCR (BG2 and P7 primers) resulted in five major clones, while six clones were shown among MRSA isolates using BG2 and P7 arbitrarily primers. Analysis of isolates distribution in the most prevalent clones indicated that the environment of the NU and ICU was the predicted source of MRSA because most of isolates from the environment and colonized patients in both units showed the same clone.

Thus, our results confirm the usefulness of combined usage of both genotypic methods (RS-PCR and AP-PCR) and AP-PCR with different arbitarily primers for epidemiological studies of MRSA.

CHAPTER I

INTRODUCTION

CHAPTER I

INTRODUCTION

1.1 *Staphylococcus aureus*

1.1.1 Background

The genus Staphylococcal (derived from the Greek *staphle*, a bunch of grapes; *kokkos*, a berry) was given its name by Ogston in 1881 when he observed grape-like clusters of bacteria in pus from human abscesses. Later on, Rosenbach isolated these microorganisms in pure cultures and owing to their name golden pigmentation were named *Staphylococcus aureus* (from the Greek *aure*, gold) (17).

1.1.2 Spectrum of Diseases Caused by *Staphylococcus aureus*

Staphylococcus aureus is one of the most notorious of all the bacterial pathogens associated with human infection, particularly the methicillin resistant *Staphylococcus aureus* (MRSA)(6, 14, 27).

Staphylococcus aureus induces a diverse spectrum of diseases associated with considerable morbidity and mortality . Presentations of infections with *Staphylococcus aureus* range from cutaneous infections, such as impetigo, boils, infections of wounds originating from prosthetic devices, to severe threatening infections, such as osteomyelitis, endocarditis and bacteremia with metastatic complications (17, 20).

People at risk for *S. aureus* infections include those with underlying diseases, newborns, trauma patients, burn patients, drug abusers and neuropenic individuals (4, 17, 20).

1.1.3 Treatment of Infections with *Staphylococcus aureus*

The period between 1946 and 1950 was the golden age for treatment of staphylococcal diseases as the bacteria was exquisitely sensitive to penicillins. This combined with the introduction of tetracyclines and macrolide compounds rendered treatment of many bacterial infections, particularly those with *S. aureus*, highly effective. Death from bacterial infection was considered to be a thing from the past. A more dispassionate view was soon to prevail as the problem of drug resistance to penicillins, mediated by the production of beta-lactamase, began to severely comprise effective first line therapies (20).

During the 1960s a semi-synthetic, broad spectrum beta-lactam compound called methicillin was introduced which was active against penicillinase producing *S. aureus*. During the following years the introduction of other semi-synthetic compounds such as oxacillin and flucoxacillin, combined with the introduction of stringent hospital infection control measures, lead to a decline in the epidemic spread of *S. aureus* strains (17, 20).

1.2 Methicillin Resistant *Staphylococcus aureus* (MRSA)

1.2.1 Background

During the 1960s many isolates of *S. aureus* were found to be resistant to the new semi-synthetic beta-lactams (methicillin, cloxacillin, flucoxacillin) which became known as methicillin resistant *S. aureus* (MRSA)(3,9,11,22). The picture became complex with many researchers showing isolates to be resistant to all classes of beta-lactams including cephalosporins. MRSA epidemics were reported in many centers in Europe and North America. The notorious MRSA had evolved, 30 years later, causing immense problems in effective treatment and is associated with considerable morbidity and mortality (20).

Initially epidemics could be controlled by aminoglycoside therapy, especially gentamicin, but during the 1970s a new wave of MRSA epidemics emerged as gentamicin resistant MRSA, which have subsequently spread all over the world. Increasing multi-resistance in these strains seems to be just a matter of introducing new antimicrobial compounds (17).

1.2.2 Vancomycin: Gold Standard in Treatment of MRSA Infections

In the early 1950s, an organism named *Streptomyces orientalis* was isolated, which produce a compound later named vancomycin. This

glycopeptide compound which destroys the integrity of the bacterial cell wall, to this day remains the first line therapy against MRSA.

Alternative therapies are extremely limited as MRSA isolates are invariably multi-resistant. Second choice therapy is usually a dual combination therapy with rifampicin and fusidic acid or minocycline. Teicoplanin is used as an alternative to this combination. Carriage of MRSA by hospital staff can be controlled by the use of mupirocin (17, 20).

1.2.3 Molecular Basis of Methicillin Resistance

Beta-lactams, such as methicillin, exert their antimicrobial effect on the susceptible organism by binding to enzymes known as penicillin binding proteins (PBPs) anchored in the cell wall. PBPs are involved in cell wall biosynthesis by controlling the production of peptidoglycan (PDG), a compound unique to bacteria. *S. aureus* has three high molecular weight and one low molecular weight PBPs (6,49). These are responsible for the polymerization of PDG chains which in *S. aureus* are cross-linked by a characteristic pentaglycal side chain. The cross-linking of PDG in the presence of a beta-lactam is reduced along with septum formation in a dose dependent manner. This alone cannot kill the organism and the actual means of cell death by action of a beta-lactam is unknown.

In MRSA, the methicillin resistance determinant *mec*, is located on an extra-chromosomal piece of DNA (plasmid) of approximately 30 kb, which has no allelic determinant in susceptible strains. This additional, *mec*-associated DNA element integrates into the staphylococcal chromosome between the *spa* and *purA* gene loci. Early epidemics of MRSA in the 1970s were due to the spread of a single isolate. Since then the *mec* determinant has entered other phylogenic lineages of *S. aureus* on several independent occasions.

The *mec A* contained within this extra-chromosomal DNA encodes for additional PBP protein PBP 2' (or PBP 2a). This PBP has a lower affinity for beta-lactam compounds than normal staphylococcal PBPs and is though to take over PBP metabolism at concentrations of beta-lactams that saturate the normal PBPs of the cell, with apparently no contribution to PDG metabolism under normal conditions of growth. PDG decreased to only 15% compared to 60% in a normal cell.

Sequence studies of *mec A* gene suggested that the *mec* gene arose by fusion of a beta-lactamase regulatory region with a structural gene of low-affinity PBP of unknown origin. The control of *mec A* expression is complex and heterogeneic.

Expression of PBP 2' is controlled by its own regulatory element but also shares the same induction system as beta-lactamases (17).

Low-level methicillin resistance in *mec*A negative strains of *S. aureus* is a biomedically and genetically diverse phenomenon, distinct from true methicillin resistance. Such resistance can arise from several sources.

1. Stepwise selection of colonies on increasing concentrations of methicillin can result in raised minimal inhibitory concentrations (MICs) to this compound. This is a result of multiple mutations in the chromosome which alter the structure of the PBPs (PB2 and PB4) and so their ability to bind beta-lacatms or to cross-link the cell wall.

2. Over production of beta-lactamase enzyme has been shown to contribute to methicillin resistance. Such hyperproduction results in a partial hydrolysis of the penicillinase resistant penicillin molecule.

3. Small colony variants of *S. aureus* can often occur in recurrent infections. Such colonies are deficient in some aspect of their electron transport system and so have impaired ATP production, which leads to a relatively inactive growing cells (17,20).

1.2.4 Types of Resistance of MRSA

1. Heterogeneous Resistance: Resistance typically is heterogeneous. Only rare cells express the resistance trait and grow in the presence of high concentration of drug. Most cells appears susceptible to relatively low, therapeutically achievable concentrations of drug. Thus, heterogeneous strains can be considered to be composed of two

populations of cells: relatively susceptible cells and highly resistant cells (9).

2. Homogenous Resistance: a majority of strains are homogeneous; i.e., cells are uniform in expression of resistance and can grow in high concentrations of drug. Thus, homogeneous strains are composed of a single population of cells , all of which tend to be highly resistant (3).

1.2.5 Types of MRSA (Epidemic MRSA versus Sporadic MRSA).

MRSA strains vary considerably in their epidemic potentials; some of them have the ability to spread widely and rapidly among patients and have been termed epidemic MRSA (EMRSA) strains (6). Once introduced into an institution these EMRSA strains are difficult to control and eradicate. This implied that for a given period these isolates were acquired by many patients during their hospitalization in the same ward (time and space clustering) and that these isolates were all genetically related (18). Other MRSA strains which are recovered from only one patient and lack the capacity to spread extensively have been termed sporadic MRSA (SMRSA) (6). Discrimination between EMRSA and SMRSA strains is important in terms of a hospital infection control policy. Detection of a discriminative marker would allow a more selective implementation of infection control measures in order to prevent dissemination of EMRSA strains within the hospital (18).

1.3 Prevalence of MRSA

The prevalence of MRSA is highly variable from country to country a round the world, and in different cities within the same country or even within a city or a hospital (5,20). In Europe, the incidence of MRSA varies from <1% in the Netherlands (0.3%), Sweden, Denmark (0.5%), and the United Kingdom (0.6%) to >30% in the southern European countries such as Spain, France (50%), and Italy (14).

The low prevalence of MRSA in the northern European countries may be due to strict hygienic measures including isolation and screening of patients from foreign hospitals and treatment of carriers.

Outbreaks in countries with a low incidence of MRSA are often initiated by the migration of patients from hospitals in countries with a high prevalence of MRSA (14).

The percentage of MRSA in the United States hospitals ranged from 15% to 45% in 1991 (4). There is a steady increase in the prevalence of MRSA isolated in hospitals in the United States over the years such that now a proximately 25% of nosocomial isolates of *S. aureus* are methicillin resistant. By 1990, MRSA strains represented 38-78% of all *S. aureus* strains isolated in tertiary hospitals in Brazil. In 1992, 50% of nosocomial *S. aureus* were MRSA in Australian hospitals.

Data from the Canadian Nosocomial Infection Surveillance Program (CNISP) show an increase in the prevalence of MRSA from

1.2% in 1995 to 5.0% in 1997. This increase was due to the success of a single clone of MRSA to spread through health-care facilities (7).

The percentage of MRSA strains accounting for *S. aureus* infections in Portugal according to the European Prevalence of Infection in intensive care study carried out in 1992 was estimated as close to 65%. The incidence of MRSA according to two national multicenter studies was estimated as 49% in 1993 and 47% in 1994. These high values are presumably related to the inappropriate use of antimicrobial drugs and to insufficient infection control measures (2, 16). In Japan, 40-60% of *Staphylococcus aureus* isolates from inpatients in general hospitals are methicillin-resistant (MRSA) (8,11).

1.4 Epidemiology of MRSA

1.4.1 Colonization of MRSA

MRSA colonization may be either transient or persistent and at a single or multiple body sites. Furthermore, patients may be colonized by multiple strains of *S. aureus*. Colonization occur in the nares, axillae, chronic wounds or decubitus ulcer surface, perineum, around gastrostomy and tracheostomy sites, in the sputum or urine. One of the most common sites of colonization in both patients and employees is the nose (anterior nares). While personnel may become colonized with MRSA, they rarely develop infections (20, 25).

1.4.2 Mode of Transmission

MRSA is transmitted primarily by contact with a person who either has a purulent site of infection, a clinical infection of the respiratory tract or urinary tract, or is colonized with the organism. Hands of personnel appear to be the most likely mode of transmission of MRSA from patient-to-patient. Several studies have demonstrated that MRSA can be present on the hands of personnel after performing activities such as wound debridement, dressing changes, tracheal suctioning, and catheter care.

Colonized and infected patients are the major reservoir of MRSA. MRSA has been isolated from environmental surfaces including floors, sinks, and work areas, tourniquets used for blood drawing, and blood pressure cuffs. Although MRSA has been isolated from environmental surfaces (e.g., floors, medical equipment), these are not the most likely source of spread (20, 25)

1.4.3 Risk Factors

Several risk factors have been identified. These include:

a. Hospitalization.

b. Multiple hospitalizations.

c. Age.

d. Multiple invasive procedures.

e. Wounds.

f. Underlying disease.

g. Administration of broad-spectrum antibiotics.

Among hospitalized patients who acquire MRSA colonization, 30-60% eventually will develop an MRSA infection such as wound infection, bacteremia, urinary tract infection, or pneumonia (20, 25,37)

1.5 Typing of MRSA

Methicillin resistant *S. aureus* (MRSA) has become a major nosocomial pathogen, causing severe morbidity and mortality at many hospitals worldwide, particularly in the intensive care settings and immunocompromised patients. Due to the frequent nosocomial outbreaks of MRSA, this microorganism requires quick , reliable characterization and identification of clonal spread (13,50). Improved typing systems are important for the rapid implementation of appropriate infection control measures and for the clinical management of MRSA infections, particularly in evaluating the efficacy of therapy for infected or colonized patients (6).

A variety of phenotypic and more recently genotypic (DNA-based) techniques have been employed for strain differentiation. Maslow et al. (1997) have characterized typing systems using five criteria: typeability, reproducibility, discriminatory power, ease of interpretation, and ease of use.

Phenotypic methods are based on biochemical, physiological and biological characteristics of the organism, whereas genotypic methods aim to detect polymorphism at the level of DNA (10).

1.5.1 Phenotypic Methods

Phenotypic characterization methods such as antimicrobial susceptibility (quantitative and qualitative antibiogram) and bacteriophage typing have been widely used in epidemiologic studies of *S. aureus* (48). The utility of these phenotypic methods is notably limited, since they may not be stably expressed under certain environmental conditions and may vary under the pressure use of antibiotics in the hospital setting (13). Although phage typing is a traditional epidemiologic typing method, it has major limitations. The method requires reagents that are not commercially available, and the data are not particularly reproducible. In addition, the discriminatory power of phage typing is often poor; furthermore, approximately 30% of *S. aureus* are nontypeable making comparisons with these isolates impossible (13).

Other phenotypic methods such as, immunoblotting, biotyping, capsule typing, and zymotyping are less frequently used.

1.5.2 DNA-Based Methods (Genotyping Methods)

The recent development of DNA-based techniques has reduced the dependence on detecting phenotypes. DNA-based typing of bacterial strains is based on the principle that epidemiologically related isolates have genetic features that distinguish them from other epidemiologically unrelated strains (13,44). Techniques used to type MRSA must be particularly discriminatory, as MRSA strains probably originate from a single clone or at least a few strain types (1, 19). Suitable typing procedures should combine typeability of all (or most) isolates, reproducibility, good discriminatory potential, easily interpretable results and practically (14,19).

Many DNA-based techniques were used to assess the relatedness of MRSA isolates, these techniques included (i) pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA (12, 19, 42), (ii) random amplification of polymorphic DNA (RAPD) (47), (iii) 16S-23S rDNA spacer amplification (RS-PCR) (6, 15, 29), (iv) protein A-gene PCR, (v) coagulase gene PCR, (vi) PCR for the characterization of the hypervariable region (HVR) adjacent to the *mecA* gene (43), (vii) restriction fragment length polymorphism (RFLP) analysis with *mec,* Tn*554, agr,* and *aph(2")-aac(6')* gene probe, (viii) multilocus enzyme electrophoresis (MLEE), (ix) plasmid profile analysis with *Eco*RI- or

*Hind*III-digested plasmid DNA, (x) Ribotyping with HindIII-digested genomic DNA and labeled rRNA (27,45)

1.5.2.1 16S-23S rDNA Amplification (Ribosome Spacer Polymerase Chain Reaction (RS-PCR))

Nucleic acid-based diagnostic assays are increasingly used in clinical bacteriology. The rapidity, specificity, and sensitivity of this technique often surpasses those offered by conventional in vitro culture and biochemical characterization.

The prokaryotic ribosomal DNA (rDNA) operon is a particularly useful target for the development of nucleic acid hybridization- and PCR-based assays. It is also has been well characterized in a significant number of important pathogens of animals and humans (15,29). Although the 16S rRNA gene has been most widely used, the 16S-23S rDNA intergenic spacer has received increased attention as a target in molecular detection and identification schemes. This approach is facilitated by previously described oligonucleotide primers complementary to sequence subunits at the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene that allow nonspecific PCR amplification of the 16S-23S spacer from a wide range of eubacteria (15,29).

Amplification of the fragments of the 16S-23S rRNA intergenic spacer region by ribosome spacer PCR (RS-PCR) can detect a significant

level of length and sequence polymorphisms at the genus species levels. Analysis of these sequences are useful in differentiating closely related members of a number of genera. Studies has suggested that PCR-amplified fragments of the intergenomic spacer regions of *Staphylococcus aureus* can produce patterns which are strain specific (6). PCR amplicon heterogeneity among 16S-23S spacer has been successfully used to differentiate bacterial species within the *Streptococcus milleri* group, to identify individual strains of methicillin-resistant *Staphylococcus aureus*, and to differentiate clinically significant species of the genus *Enterococcus* (15).

1.5.2.2 Arbitrarily Primed PCR (AP-PCR)

AP-PCR involves random amplification of the target DNA using a single primer that does not have any known homology to the target sequence. Amplification is conducted at low annealing temperatures, which allows mismatches and thus permits arbitrary primer sequences to bind nonspecifically to DNA template. Amplicons are generated whenever two correctly oriented copies of the primer are close enough for the PCR to proceed efficiently. Differences in the distance between primer-binding sites lead to synthesis of amplicons, that differ in length. These differences can be detected by gel electrophoresis (10, 24)

In terms of typeability, reproducibility, and discrimination power, the results obtained by AP-PCR in epidemiological investigation of

MRSA outbreaks were comparable with those obtained by PFGE (14), which is currently considered to be a reference typing method. However, PFGE analysis is costly and technically demanding, and it still requires interlaboratory standardization (12 ,21, 22, 23).

1.6 Aims of the Study

Epidemiological infections due to MRSA continue to be a major problem in many countries, thus epidemiological studies of MRSA are necessary to identify possible clusters of cases, which is important in terms of a hospital infection control policy. Detection of a discriminative marker would allow a more selective implementation of infection control measures in order to prevent dissemination of MRSA in hospitals.

In Palestine, clinical isolates of *Staphylococcus aureus* resistant to methicillin and other antibiotics are present, however data describing their prevalence, patterns of resistance, genetic and epidemiological relatedness have not been investigated. The aims of this study are to:

1. Determine the resistance rates of MRSA strains against several antibiotics.
2. Evaluate three epidemiological typing systems, RS-PCR, AP-PCR, and antibiogram for their performance and convenience in differentiating MRSA strains.
3. Evaluate the AP-PCR typing system by using two separated arbitrary primers; P7 and BG2.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

2.1 MRSA Isolates

A collection of 44 MRSA isolates, previously isolated from Rafidya hospital, northern Palestine (40 isolates, 91%) and three hospitals located in southern Palestine (4 isolates, 9%) between February and May 1998, was included in this study. Information about the origin of the isolates is presented in Table 3. Isolates were recovered from infected patients (7 isolates, 15.9%), colonized patients (21 isolates, 47.7%) and hospital environment (16 isolates, 36.4%).

2.2 Identification of *S. aureus*

Carriage of *S. aureus* was determined by culturing of samples on blood agar plates, plates were incubated for 24 h at 35°C and observed for the growth of suspected *S. aureus* colonies (32).

Standard microbiological methods for identification of *S. aureus* included colony morphology, gram staining, growth on mannitol salt agar, positive catalase slide test, positive DNAse test, and positive coagulase tube test (overnight colony suspended in 0.5ml of 1:10 diluted plasma incubated at 37°C for 2h) were employed (4, 18, 20). After the identitification of the isolates was confirmed, they were stored at -85 °C

in a brain heart infusion broth-glycerol mixture (3:10; vol/vol) in freezer vials pending further analysis (32).

2.3 MRSA Determination

Screening for methicillin resistant *S. aureus* (MRSA) isolates were detected by two standard methods:

1. Disk-agar diffusion susceptibility method with 1 µg oxacillin disks placed on Muller-Hinton agar (Difco) with 4% NaCl supplementation. The inoculum size was adjusted to a final concentration of 10^5 CFU/ml. The zone of inhibition were determined after an incubation of 24 h at 35°C. Methicillin resistance was defined according to the National Committee for Clinical Laboratory Standards (NCCLS) (31) breakpoints. A zone diameter of inhibition <10 mm was considered as indicative of resistance (33).

2. Growth of isolates on Muller-Hinton agar (Difco) supplemented with 4% sodium chloride (NaCl) and 6 µg of oxacillin per ml after incubation at 35°C for 24 h (33,39).

2.4 Antimicrobial Susceptibility Testing "Antibiogram"

The susceptibilities of all isolates to different antimicrobial agents were tested by the disk agar diffusion method on Muller-Hinton agar (Difco) as standardized by the National Committee for Clinical Laboratory Standards (NCCLS) document M2-A5 (31).

The following antimicrobial agent-containing disks and concentrations were used : ampicillin, 10 μg; refampicin, 2 μg; ciprofloxacin, 5 μg; chloramphenicol,30 μg; clindamycin, 10 μg; erythromycin, 5 μg; gentamycin, 10 μg; trimethoprim-sulfmethoxazole, 25 μg; tetracycline, 30 μg; vancomycin, 30 μg; novobiocin, 5 μg; oxacillin, 1 μg; oflaxacin, 5 μg; amikacin, 30 μg, and imipenem, 10 μg. (Oxoid). Inoculum size was adjusted to deliver a final concentration of 10^5 CFU/ml, where a direct colony suspension of the culture equivalent to a 1.0 M McFarland standard was prepared in a normal saline (0.9% NaCl) and the plates were cultured for *S. aureus*. The results were recorded after 24 h of incubation at 35°C (33,40).

2.5 Genotyping of Methicillin Resistant *Staphylococcus aureus*

2.5.1 Preparation of Bacterial DNA Templates

5-10 colonies, picked from 18 h cultures on Muller-Hinton agar plates (Difco) were suspended in 100μl of lysing solution containing 125μg/ml lysostaphin (Sigma), 1 mg/ml lysozyme (Sigma) in TE buffer (10 mM Tris, 0.1 mM EDTA, [pH 8]). The suspension was incubated at 37°C for 60 min., after that, 10 μl of proteinase K (10 mg/ml) (Sigma) was added and incubation was continued for additional 30 min. The suspension was then heated at 97 °C for 10 min in a thermocycler machine (Stuart Scientific), centrifuged and stored in the freezer (-20°C) for amplification.

2.5.2 Ribosome-Spacer PCR (RS-PCR)

2.5.2.1 RS-PCR Amplification Procedure

Primer G1 (5'-GAAGTCGTAACAAGC-3')(Eurogenetec), which was selected from a highly conserved region in the 16S RNA gene, is located about 30-40 nucleotides upstream from the spacer region.

Primer L1 (5'-CAAGGCATCCACCGT-3') (Eurogenetec) contained the most conserved 23S sequence immediately following the spacer and is located about 20 nucleotides downstream from the spacer boundary.

Primers for both the 16S and 23S regions were limited to a length of 15 nucleotides because of variations in sequence beyond these highly conserved regions.

PCR amplification was carried out in 25 µl (total volume) mixture containing 5 µl of the prepared DNA template and 20 µl of a solution which contained 5 pmol of primer G1, 5 pmol of primer L1, 14.5 µl of deionized, distilled water, 2.5 µl of 10X PCR buffer (500 mM KCl, 100mMTris HCl [pH 9.0]; Promega Corporation, Madison, Wis.), 250 mM (each) dATP, dCTP, dGTP, and dTTP (Promega Corporation), 2 µl of 25 mM $MgCl_2$ (Promega Corporation), and 1 U of Taq DNA polymerase (Promega Corporation). PCR mixture was overlaid with the same volume (25µl) of mineral oil.

PCR amplification was performed with a Stuart Scientific thermocycler. After an initial denaturation step of 2 min at 94°C, a total of 34 cycles of amplification was performed using the following thermal profile: 1 min at 94°C (denaturation), 1 min at 45°C (annealing), and 1 min at 72°C (elongation). The last cycle was followed by a 5 min extension step at 72°C. To check on possible contamination, a negative control containing distilled water instead of template DNA was included in each experiment. Amplified DNA was stored at -20°C.

2.5.3 PCR-Mediated DNA Fingerprinting (Arbitrarily Primed PCR [AP-PCR])

2.5.3.1 AP-PCR Amplification Procedure

The AP-PCR was carried out in 25 µl reaction volumes. Each reaction mixture contained 5 µl of the prepared DNA template and 20 µl of a solution which contained 14.5 µl of deionized, distilled water, 2.5 µl of 10X PCR buffer (500 mM KCl, 100mMTris HCl [pH 9.0]; Promega Corporation, Madison, Wis.), 250 mM (each) dATP, dCTP, dGTP, and dTTP (Promega Corporation), 2 µl of 25 mM $MgCl_2$ (Promega Corporation), and 1 U of Taq DNA polymerase (Promega Corporation).

Two different primers were included in the typing assays. The designations and sequences of the primers were as follows: BG2, 5'-GGTTGGGTGAGAATTGCACG-3'; and P7, 5'-CAA GGC ATC CAC

CGT-3')(Eurogenetec) (41). All primers were applied in separate assays (5 pmol of primer per PCR mixture).

PCR mixture was overlaid with the same volume of mineral oil.

PCR amplification was performed with a Stuart Scientific thermocycler. After an initial denaturation step of 4 min at 94°C, a total of 35 cycles of amplification was performed using the following thermal profile: 1 min at 94°C (denaturation), 1 min at 42°C (annealing), and 1 min at 72°C (elongation). The last cycle was followed by a 10 min extension step at 72°C. To check on possible contamination, a negative control containing distilled water instead of template DNA was included in each experiment. Amplified DNA was stored at -20°C.

2.5.4 Separation of Amplified Products by Electrophoresis

Amplification products were separated by electrophoresis in 8-mm-thick 2% agarose gels (Appligene) with a Gencons apparatus. Gels were run in 1X Tris-borate-EDTA (TBE) at a constant current of 100mA and a voltage of 120V for about 2 hours prior to electrophoresis, samples were mixed with 5 µl of DNA loading buffer (50% glycerol in water and 0.8 mg of bromophenol blue per ml). Then 20 µl of the amplified material was loaded on the gel, and a 5 µl of a molecular weight marker (DNA smart Ladder, MW 1700-02)(Eurogenetec) was run in parallel with the PCR samples on each every eight lanes. Gels were stained before electrophoresis by addition of 5 µl of ethedium bromide (10mg/ml)

(Sigma) to a total volume of 100 ml of 1X TBE and 2 g of agarose. The gels were photographed on a 392 nm wavelength transilluminator with a Polaroid MP4 Landcamera and Polaroid 57 high speed films, with an exposure time 4.5 and lens aperture 8.

2.6 Analysis of PCR Profiles

Analysis of PCR profiles was performed by visual inspection of photographs of ethedium bromide stained gels. The total numbers of visible bands were counted for each isolate, and patterns were compared according to the number and size of the resultant bands; banding pattern were digitized with a Hewlett-Packard scanjet scanner.

Banding patterns from the majority of epidemiologically related isolates that appeared identical in size and number of bands were considered to represent the same strain, which was designated in Latin numbers (I, II, III, etc...) for RS-PCR patterns, capital letters (A, B, C, etc...) for BG2-PCR, and small letters (a, b, c, etc...) for P7-PCR. Coefficient of similarity (Dice coefficients of correlation) was determined using the following formula , CS = number of shared bands × 2 × 100/ total number of bands in the two samples (18,26,28,30).

CHAPTER III

RESULTS

CHAPTER III

RESULTS

3.1 Susceptibility to Antimicrobial Agents

All MRSA isolates were resistant to ampicillin and oxacillin. The majority were also resistant to clindamycin (95.5%), erythromycin (86.4%), tetracyclin (72.2%) and trimethoprime (61.4%), rendering these antibiotics ineffective as therapeutic agents against MRSA. Resistance to vancomycin and refampicin was not observed. Resistance to the remaining 7 antibiotics ranged from 6.8% to 50% (Figure 1). Around 36.4% of MRSA isolates were resistant to 8 or more antibiotics, 9.1%, 11.4%, 20.5%, 6.8%, 9.1%, and 6.8% were resistant to 6, 5, 4, 3, 2, and 1 antibiotic (s), respectively (Table 1).

Antibiotic resistance patterns encountered among the isolates are shown in Table 2. Seventeen distinct patterns are identified, 6 had antibiotype 1; 6 had antibiotype 8; 4 had antibiotype 14; 3 had antibiotypes (5, 6, 9, 12, and 13); 2 had antibiotypes (2, 3, 7, and 15). Antibiotypes 4, 10, 11, 16, and 17 were represented by a single MRSA isolate. The distribution of MRSA isolates with respect to their antibiotypes is shown in Figure 2.

3.2 PCR Analysis

3.2.1 RS-PCR

The RS-PCR assay of MRSA isolates with primers L1 and G1 yielded amplicons ranged in size from 300 bp to 800 bp, with 1 to 6 fragments being resolved per isolate. Eleven RS-PCR patterns were identified, designated I through XI. Four isolates (9%) were non typeable. The most frequent patterns were I (9 isolates) and II (7 isolates). A single, two, three, or four isolates represented the remaining patterns. RS-PCR patterns and a schematic preview for each pattern, including the number of isolates are shown in Figures 3 and 4.

3.2.2 AP-PCR (BG2 Primer)

Genomic DNA amplification of MRSA isolates using BG2 arbitrarily primer resulted in fifteen different distinct DNA banding patterns. Six (13.6%) of MRSA isolates were non typeable. The number of amplified DNA fragments ranged between 1 to 7 distinct bands in each pattern. Fragment size ranged from 300-800 bp (Figures 5 and 6).

BG2 pattern A and B were the most prevalent and were represented by 9 and 7 isolates, respectively. A single, two, three or five isolates represented the remaining patterns. AP-PCR patterns and a schematic preview for these patterns, including number of isolates in each are shown in Figures 5 and 6.

3.2.3 AP-PCR (P7 Primer)

Genomic DNA amplification of MRSA isolates using P7 arbitrarily primer resulted in fourteen different distinct DNA banding patterns. Two (4.5%) of MRSA isolates were non typeable. The number of amplified DNA fragments ranged between 1 to 11 distinct bands in each pattern. Fragment size ranged from 300 -900 bp.

P7 patterns (a and b) were the most prevalent among the P7 genotypes, and were represented by 12 and 5 isolates, respectively. The number of isolates in rest of the main genotypes ranged between 1 and 4.

P7 AP-PCR patterns and a schematic preview for these patterns, including number of isolates are shown in Figures 7 and 8.

3.3 Typeability and Discriminatory Power the Genotyping Methods

Typeability refers to the ability of a test to provide an ambiguous results for each isolate examined; non typeable isolates are those that produce a null or ambiguous results. Accordingly, four of the 44 MRSA (9%) isolates were non typeable by RS-PCR. AP-PCR using BG2 and P7 primers failed to type 6 (13.5%) and 2 (4.5%) of the 44 MRSA isolates, respectively.

Discriminatory power refers to the ability of a technique to differentiate among unrelated isolates. In our study, seven isolates (A5, A11, A12, C37, R 68, R 69, and R 95) were considered epidemiologically

unrelated because either they originated from different geographic areas (n=4), or they had been sampled from recently admitted patients with no link to other known MRSA carrier and/or infected patients in the hospital (n=3) (Table 3). These isolates were used to evaluate the discriminatory power for each of the used typing methods. RS-PCR typed them into 4 different spacer types (2 in I and V, 1 in III and VIII, and 1 nontypeable). BG2 AP-PCR typed them into 5 different BG2 types (2 in type D, 1 in types A, B, N, G, and 1 nontypeable); P7 AP-PCR typed them into 6 different types (2 in type b, 1 in types c, d, j, k, and n). Discriminatory power increased in the order RS-PCR < BG2 AP-PCR < P7 AP-PCR yielding 4, 5, and 6 different types, respectively.

3.4 Correlation of the Four Used Typing Methods

The 37 epidemiologically related isolates, were typed into 16 antibiotypes, 11 spacer types, 13 BG2 types, and 12 P7 types.

RS-PCR showed two predominant patterns (I and II) among the 37 epidemiologically related isolates of Rafidya hospital. Each pattern was represented by 7 isolates. The two patterns were genetically related , with coefficient of similarity " CS" of 86%. Consequently, the two patterns were considered as one distinct epidemiological clone. Of this clone, 8 isolates were collected from colonized cases in the neonatal and intensive care units during their hospitalization and 6 were environmental

isolates from both units. All of the environmental epidemic isolates were RS-PCR type I, while 7 (87.5%) of the colonized isolates were represented by type II. From this observation we conclude that isolates of this clone have the capacity to spread between patients and accordingly considered epidemic.

Of the 14 isolates of the major RS-PCR clone, BG2-PCR further divided 9 (64.3%) of the these isolates into 3 BG2 types (type B , 4 isolates; type C, 3 isolates; and type A, 2 isolates). The remaining isolates were typed as sporadic. While, P7-PCR further divided 9 (64.3%) of the epidemic isolates into 2 P7 types (type a, 6 isolates; and type f, 3 isolates). The rest of isolates were also typed as sporadic.

All of RS-PCR major clone isolates were resistant to ampicillin, oxacillin, chloramphenicol, and clindamycin and were susceptible to impenim, vancomycin, and oflaxacin which showed no correlation with the results of RS-PCR and AP-PCR.

The remaining RS-PCR types (III through XI) were genetically unrelated to the epidemic strain, with a coefficient of similarity of 85% or less.

The isolates of these patterns were considered as sporadic , since their isolation was infrequent.

3.5 Cluster Analysis

The combination of the RS-PCR and AP-PCR patterns with the two primers (BG2 and P7) allowed us to define 27 clones among the epidemiologically related isolates of Rafidya hospital (Table 3). The high degree of heterogeneity should be considered together with the clear evidence of 5 major clones among the MRSA isolates. I:B:a and II:C:a clones were the most common clones including 3 isolates each. Two isolates of clone I:B:a were collected from the environment of the neonatal unit (NU) where the other was collected from a colonized patient in the intensive care unit (ICU). Isolates of clone II:C:a were collected from colonized patients in the neonatal unit. Two isolates were found in the following clones : III:A:a, II:A:f and V:C:c. The remaining clones were considered as sporadic since they were found only in a small number of isolates and all of them were heterogeneous.

Table 1. Percentage of multidrug resistance.

No. of antibiotics	No. of isolates	Resistance (%)
10	8	18.2
8	8	18.2
6	4	9.1
5	5	11.4
4	9	20.5
3	3	6.8
2	4	9.1
1	3	6.8

Total = 44

Table 2. Methicillin Resistant *Staphylococcus aureus* (MRSA) antibiogram types retrieved in this study [a]

Type	No. of Isolates	Susceptibility to the following antibiotics:														
		Am	Ox	Ci	Ch	Cl	Er	Ge	Tr	Te	No	Of	Ak	Im	Re	Va
1	6	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S
2	2	R	R	R	R	R	R	R	R	R	S	R	R	R	S	S
3	2	R	R	R	R	R	R	R	R	R	S	R	R	S	S	S
4	1	R	R	R	S	R	R	R	R	R	S	S	S	S	S	S
5	3	R	R	R	S	R	R	R	R	R	S	R	S	R	S	S
6	3	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S
7	2	R	R	S	R	S	R	S	R	R	R	S	S	S	S	S
8	6	R	R	S	S	R	R	S	S	R	R	S	S	S	S	S
9	3	R	R	S	R	R	R	S	S	R	S	S	S	S	S	S
10	1	R	R	S	R	R	R	R	R	S	S	R	S	S	S	S
11	1	R	R	R	S	R	R	S	R	R	S	R	S	S	S	S
12	3	R	R	S	S	R	R	S	R	S	S	S	S	S	S	S
13	3	R	R	S	R	R	S	R	R	R	S	S	S	S	S	S
14	4	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S
15	2	R	R	R	S	R	R	R	R	R	R	R	S	S	S	S
16	1	R	R	R	S	R	R	R	S	S	R	R	S	S	S	S
17	1	R	R	S	S	R	R	R	R	R	R	S	R	R	S	S

[a] Am, ampicillin; Ox, oxacillin; Ci, ciprofloxacin; Ch, chloramphenicol; Cl, clindamycin; Er, erythromycin; Ge, gentamycin; Tr, trimethoprime; Te, tetracyclin, No, novobiocin; Of, oflaxicin; Am, amikacin; Im, imipenem; Re, refampicin; Va, vancomycin; R, Resistant; S, Susceptible.

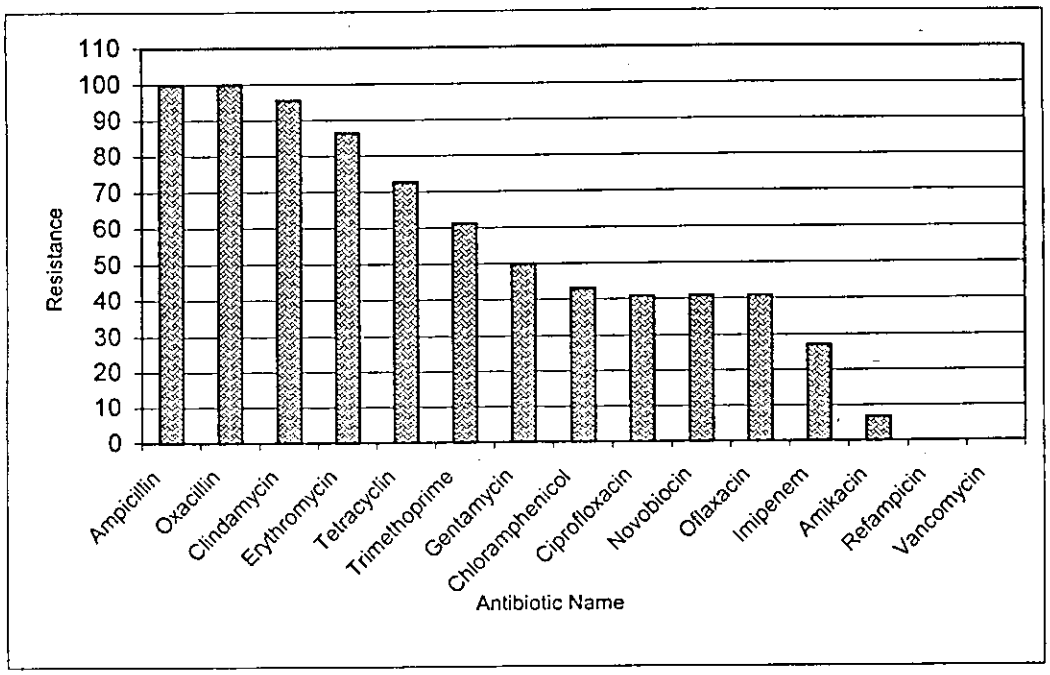


Figure 1. Histogram represents the resistance percentage of 44 MRSA isolates for each of the used antimicrobial agents.

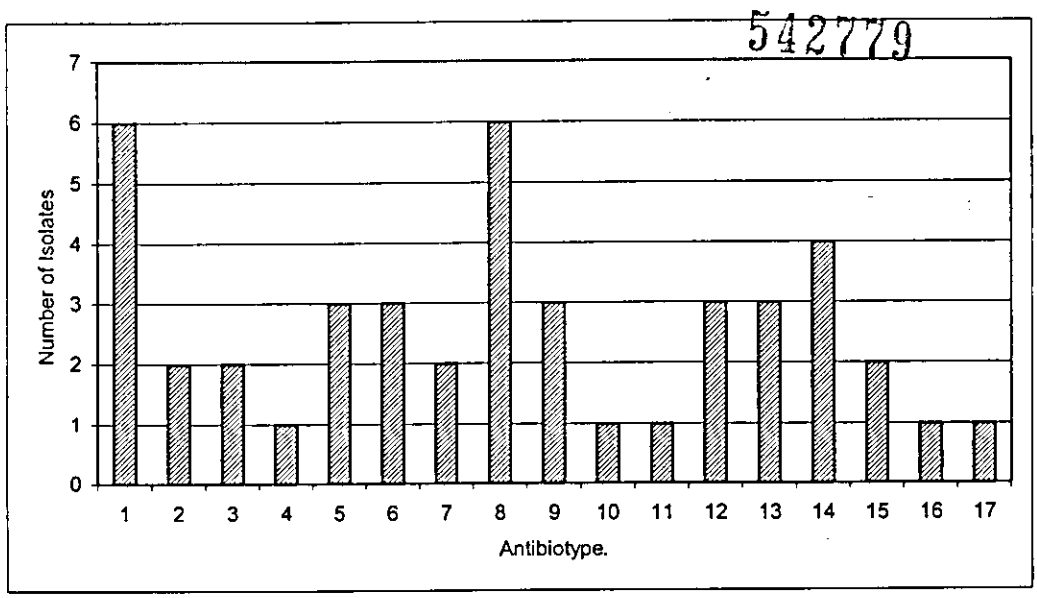


Figure. 2. Histogram represents the distribution of 44 MRSA isolates on 17 antibiotypes, (1through 17).

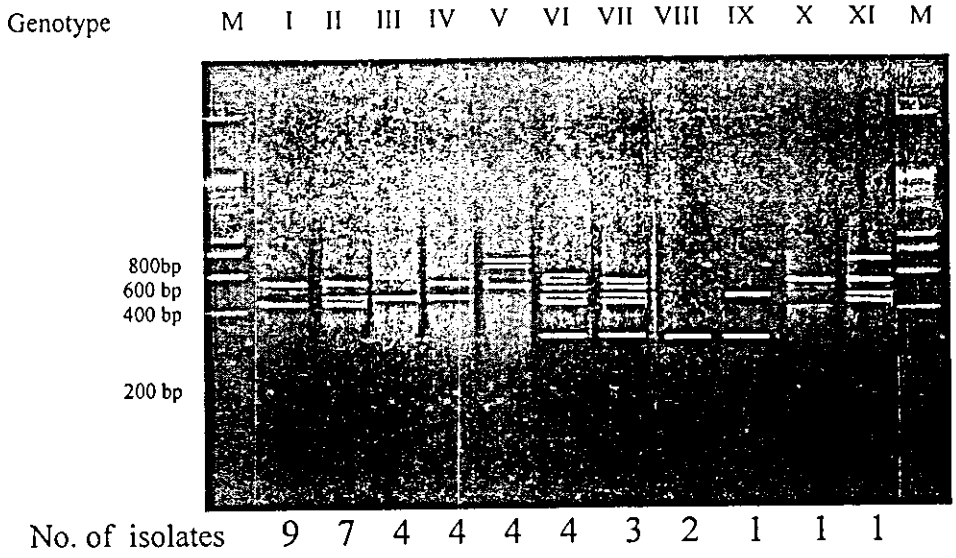


Figure 3. Representative RS-PCR patterns, including the number of isolates in each. Molecular weight marker (DNA smart ladder) are in lanes 1 and 13. Lanes 2 to 12 each contain a different isolate.

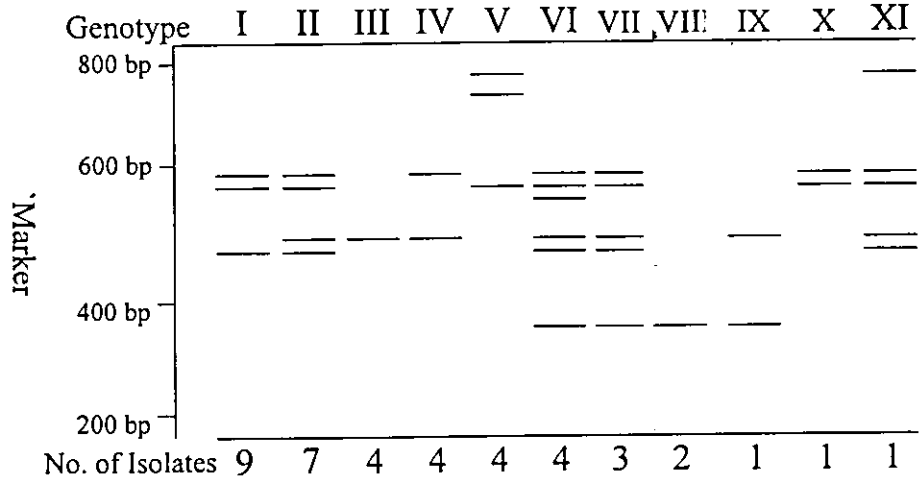


Figure 4. Schematic presentation of RS-PCR patterns, including number of isolates in each.

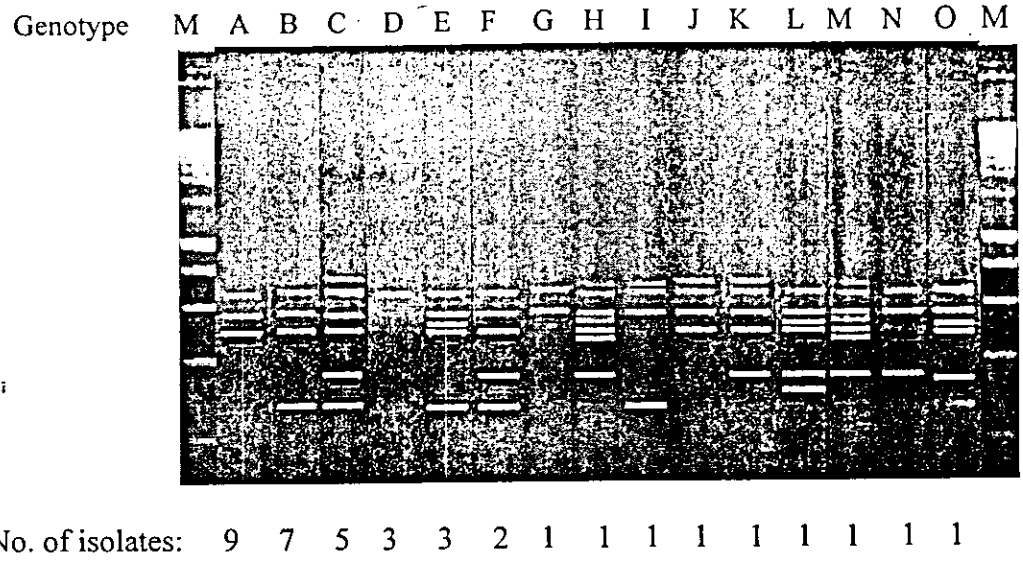


Figure 5. Representative BG2 AP-PCR patterns, including the number of isolates in each. Molecular weight marker (DNA smart ladder) are in lanes 1 and 17. Lanes 2 to 16 each contain a different isolate.

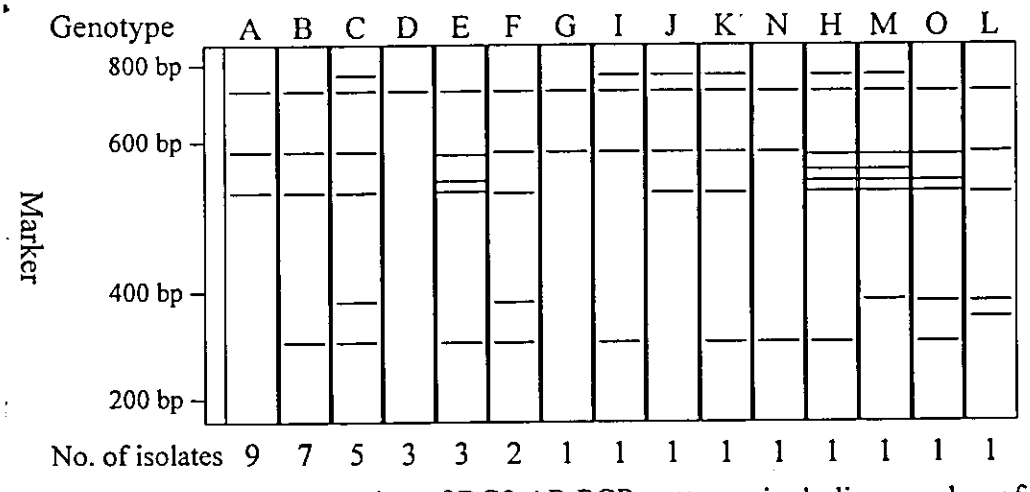


Figure 6. Schematic presentation of BG2 AP-PCR patterns, including number of isolates in each.

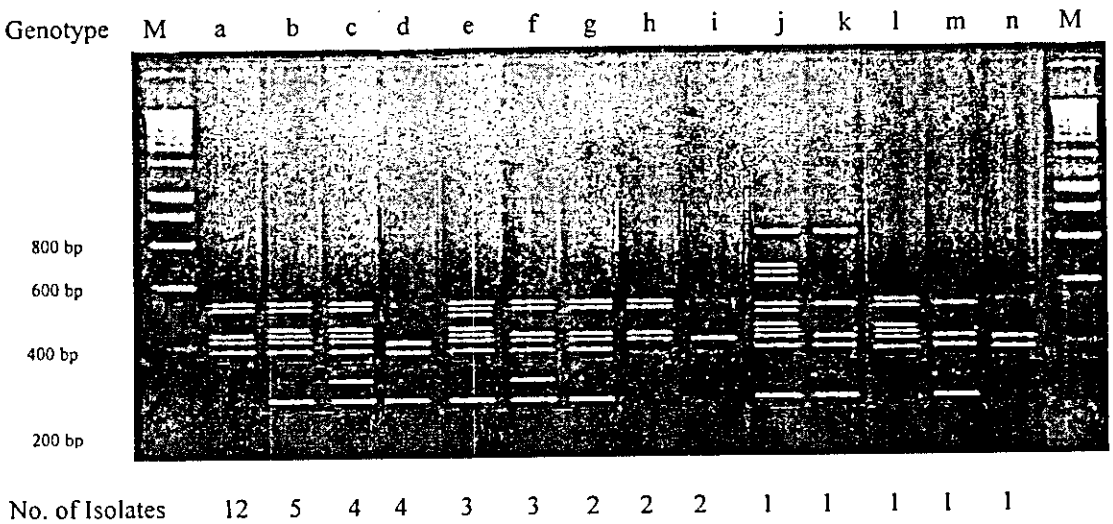


Figure 7. Representative P7 AP-PCR patterns, including the number of isolates in each. Molecular weight marker (DNA smart ladder) are in lanes 1 and 13. Lanes 2 to 12 each contain a different isolate.

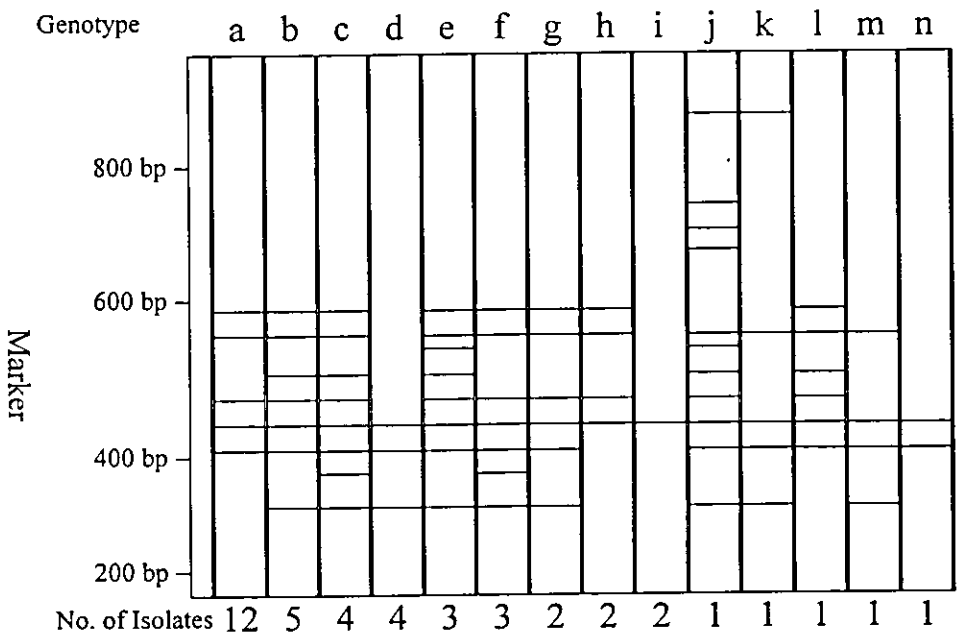


Figure 8. Schematic presentation of RS-PCR patterns, including number of isolates in each.

Table 3: MRSA strains used in the study and their RS-PCR and AP-PCR genotypes.

Isolate	Source		RS-PCR (11)[b]	AP-PCR BG2 (15)	AP-PCR P7 (14)	Clonal type RS:BG2:P7 (27)
	Site	Ward[a]				
R 715	Environment	ICU	III	A	A	III:A:a
R 313	Environment	ICU	III	A	A	III:A:a
R 095*	Blood	Outpatient	VI	A	C	VI:A:c
R 588	Environment	ICU	VI	A	C	VI:A:c
R 519	Environment	NU	VIII	A	E	VIII:A:e
R 464	Groin	NU	II	A	F	II:A:f
R 760	Nose	ICU	II	A	F	II:A:f
R 316	Environment	NU	I	B	A	I:B:a
R 442	Environment	NU	I	B	A	I:B:a
R 730	Nose	ICU	I	B	A	I:B:a
A 005	Urine	ND	VIII	B	D	VIII:B:d
R 534	Environment	NU	VI	B	E	VI:B:e
R 578	Environment	NU	I	B	H	I:B:h
R 271	Umbilicus	NU	II	C	A	II:C:a
R 763	Umbilicus	NU	II	C	A	II:C:a
R 347	Groin	NU	II	C	A	II:C:a
R 272	Nose	NU	V	C	C	V:C:c
R 561	Prostate	HCW	V	C	C	V:C:c
R 069	Pus	Outpatient	I	D	B	I:D:b
R 386	Nose	ICU	III	D	D	III:D:d
C 037	Umbilicus	ND	V	D	J	V:D:j
R 479	Umbilicus	NU	III	E	A	III:E:a
R 731	Groin	NU	IV	E	B	IV:E:b
R 322	Environment	ICU	IV	E	I	IV:E:i
R 668	Environment	NU	VII	F	A	VII:F:a
R 669	Environment	ICU	I	F	F	I:F:f
A 011	Urine	Outpatient	V	G	K	V:G:k
R 756	Nose	NU	VII	H	G	VII:H:g
R 422	Environment	NU	I	I	D	I:I:d
R 647	Nose	ICU	IX	J	A	IX:J:a
R 639	Environment	NU	IV	K	G	IV:K:g
R 769	Groin	NU	VII	L	A	VII:L:a
R 655	Nose	NU	VI	M	B	VI:M:b
R 068	Pus	Outpatient	I	N	B	I:N:b
R 727	Nose	ICU	IV	O	H	IV:O:h
R 315	Environment	NU	I	NT	L	***
R 663	Nose	NU	II	NT	I	***
R 202	Nose	NU	II	NT	NT	***
R 041	Nose	HCW	X	NT	NT	***
R 371	Nose	NU	NT	A	D	***
R 657	Environment	NU	NT	A	E	***
R 443	Environment	NU	NT	B	M	***
A 012	Urine	ND	NT	NT	N	***
R 522	Environment	NU	XI	NT	B	***

[a] Ward designations are as follows: NU, Neonatal Unit; ICU, Intensive Care Unit; HCW, Health Care Worker, and NT, Not Typeable.

[b] Numbers in parenthesis represent the numbers of types in each typing system.

* Selected isolates were unrelated.

CHAPTER IV

DISCUSSION

CHAPTER IV

DISCUSSION

Staphylococcus aureus, especially methicillin resistant (MRSA) has emerged, globally, as a major nosocomial pathogen in the early 1960s. The rapidity with which methicillin resistance developed in Europe after the introduction of methicillin and the wide spread of the organism throughout the world raises the question of the management of infected patients in hospitals in many parts of the world (6, 20). Moreover, an increase in the frequency of MRSA strains resistant to multiple antibiotics in both large and small hospitals complicated the problem (32).

In Europe, the incidence of MRSA varies from less than 1% in The Netherlands, Sweden, Denmark, and the United Kingdom to more than 30%in the southern Europe countries such as Spain, France, and Italy (14,46). The incidence is particularly high in Japan, being 60% nationwide; and as high 90% in individual hospitals. Several studies carried out in the United States found that , 15% to 45% of nosocomial isolates of *Staphylococcus aureus* are methicillin resistant (4). Different rates among various countries can be affected by variation in patient populations, hospital care practices, infection control activities and the

nature of staphylococcus strains (34). Strains may vary considerably in their epidemiological potentials, and those strains that have been known to spread widely and rapidly among patients should be designated epidemic *S. aureus* strains (EMRSA) (6, 14,38).

As in previous reports, our bacterial collection of MRSA strains were often resistant to clindamycin (95.5%), erythromycin (86.4%), tetracyclin (72.2%), trimethoprim (61.4%), and gentamycin (50%). In addition, resistance to at least 3 drugs was found in 78% of the isolates (see Table1). Resistance to erythromycin and clindamycin may be due to the acquisition of a new genetic material, most likely the transposable Tn*554* (9). Although it is probably an infrequent event, regional and distant spread of methicillin resistant clones with variable selective pressure that promote sequential development of resistant determinants is likely to play a major role.

An observation of primary concern is the high incidence of resistance to gentamycin (50%). This probably reflects the fact that such antibiotic has been extensively used in Rafidya hospital , especially in the neonatal units as prophylactic and therapeutic agent (personal communication). Although ciprofloxacin is not well introduced into clinical practice in our hospitals, 40.9% of our isolates were resistant. It appears that the extensive usage of broad-spectrum antibiotics induces MRSA strains of different genotypes to undergo multiple mutation and

acquire resistance genes to ciprofloxacin. Thus, limiting the therapeutic value of this agent in future control of MRSA (21).

Among the antibiotics tested, refampicin and vancomycin behave as the most potent agents. This observation may provide the rational for alternative therapy that can be used in patient populations that are likely to be infected with MRSA. Resistance to more than one antimicrobial agents might be due to multiple mutations on the chromosomal and plasmid genes as aresult of high use of antibiotics.

Accurate epidemiological typing is of primary importance for the identification of MRSA clones found in a hospital and for enabling sources and routs of transmission to be identified and controlled. At present, several phenotypic and more recently genotypic techniques have been employed for strain differentiation (10). The ideal typing system should have the following criteria: typeability; reproducibility, discriminatory power, ease of interpretation, and ease of use.

In our study, typeability of the used typing methods seems to be within the acceptable range (14), the best typeable method was the P7 AP-PCR (95.5%). The discriminatory power is the ability to distinguish unrelated isolates; it is determined by the number of types identified by the method and the relative frequencies of these types. In some studies, epidemiological typing methods were done with a too small number of unrelated strains for the evaluation of the discriminatory power of the

methods (12). Despite the limited number of unrelated isolates, the found ID (Dice coefficients of correlation) values were acceptable (23).

The finding of seventeen distinct antibiotypes among our MRSA collection clearly indicates that the use of resistance to antibiotics is poor and unstable marker. This might be due to the fact that drug resistance is often plasmid borne and may be gained or lost over time (6). In our study, most of the strains exhibited antibiogram pattern which did not correlate with the observed genotypic clusters (see Table 3).

As a general rule, poor correlation has been noticed between antibiotyping and genotypic typing methods in MRSA (36). Thus, it is reasonable to suggest that multiple antibiogram types do not necessarily indicate multiple epidemic strains, since antibiotic resistance is greatly influenced by drug usage. Furthermore, it is likely that within a hospital of epidemic MRSA, isolates of this organism are more likely to be genetically related (36).

Since DNA-based typing techniques of bacterial strains was shown to be more accurate in differentiating between epidemiologically related and unrelated strains, we used two types of PCR (RS-PCR and AP-PCR) to define MRSA relatedness and to detect epidemic strains.

Amplification of the 16S-23S rRNA intergenic spacer regions by ribosome spacer PCR (RS-PCR) was shown to be useful in differentiating closely related strains where it produces patterns which are strain specific

(15). In our study, application of this technique generated eleven RS-PCR distinct patterns (I through XI) among the MRSA isolates. The number of DNA fragments in each pattern ranged between 1 to 6, ranging in size from approximately 300 to 800 bp. Different patterns had a number of shared bands which may indicate for the presence of conserved intergenic spacer region. The most prevalent spacer types were type I and II which were represented by 22.5% and 17.5% of isolates, respectively (see Figures 3 and 4). Out of spacer type I isolates, 5 isolates were from the environment of the NU and the rest were from the environment and colonized patients in the ICU of Rafidya hospital. It is most likely that the environment of the NU was the original source of MRSA, while the potential reservoir could be the transiently colonized hands of the health care workers. All of spacer type II isolates were collected from the colonized patients in the NU.

Genomic DNA amplification of MRSA isolates using BG2 and P7 arbitrarily primers (AP-PCR) resulted in fifteen and fourteen different distinct DNA banding patterns, respectively. This was confirmed by the results of genomic DNA amplification using BG2 and P7 arbitrarily primers.

Combination of RS-PCR and AP-PCR (BG2 and P7 primers) resulted in five major clones (Table 3). The most prevalent clones were I:B:a, II:C:a (3 isolate each), III:A:a, II:A:f, and V:C:c (2 isolates each),

the rest of clones were represented by only one isolate each. Two isolates of clone I:B:a were collected from the environment of the neonatal unit (NU) where the other was collected from a colonized patient in the intensive care unit (ICU). Isolates of clone II:C:a were collected from colonized patients in the neonatal unit. These results support the previous conclusion that the environment of NU and ICU is the source of MRSA.

Six BG2:P7 clusters were shown among MRSA isolates. The most prevalent clusters were B:a (3 isolates) and C:a (3 isolates), the remaining clusters were represented by 2 isolates in each. Analysis of isolates distribution in the most prevalent clusters indicated that the environment of the NU and ICU was the predicted source of MRSA because most of isolates from the environment and colonized patients in both units showed the same clone.

Thus, our results confirm the usefulness of combined usage of both genotypic methods (RS-PCR and AP-PCR) for epidemiological studies of MRSA.

The observed high degree of heterogeneity among MRSA clones, using the genotyping methods, is most likely associated with multidrug resistance pressure selection. The unwise and misuse of drugs in our hospitals may induces MRSA isolates to undergo multiple mutations and acquire new genetic makeup.

RECOMMENDATIONS

1. Antibiotic resistant MRSA, especially those with multiple resistance, are increasingly isolated and are a serious problem in Palestine indicating the need for national surveillance programs to generate accurate local antimicrobial susceptibility data and studying alternative antibiotic therapies and a database for the antibiotics used in Palestine.
2. Combination of results for more than one genotyping method is more powerfull in differentiating between epidemiologically related and unrelated MRSA strains.
3. Wise use of antibiotics based on monitoring programs in the health care systems will drastically decrease the incidence of multidrug resistant MRSA isolates.
4. Quarterly routine screening for MRSA should be implemented in order to evaluate the size of the problem and the spread of such strains.

REFERENCES

1. **Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller.**1995. Pulsed-Field Gel Electrophoresis as a Replacement for Bacteriophage Typing of *Staphylococcus aureus*. J. Clin. Microbiol. **33**:551-555.
2. **Couto, I., J. Melo-Cristono, M.L. Fernandes, T. Gracia, N. Serrano, M. J. Salgado, A. Torres-Pereira, I. S. Sanches, and H. De Lencastre**. 1995. Unusually Large Number of Methicillin-Resistant Staphylococcus aureus Clones in a Portuguese Hospital. J. Clin. Microbiol. **33**:2032-2035.
3. **Champers, H. F.**1988. Methicillin-Resistant Staphylococci. Clin. Microbiol. Rev. **1**:173-186.
4. **Santos, K. R. N., L. M. Teixeira, G. S. Leal, L. S. Fonseca, and P. P. G. Filho**. 1999. DNA Typing of Methicillin-Resistant Staphylococcus aureus: Isolates and Factors Associated with Nosocomial Acquisition in Two Brazillian University Hospitals. J. Med. Microbiol. **48**: 17-23.
5. **Cookson B.** 1995. Aspects of the Epidemiology of MRSA in Europe. J. Chemother. **3**:93-98.
6. **Kumari D. N. P., V. Keer, P. M. Hawkey, P. Parnell, N. Joseph, J. F. Richardson, and B. Cookson.** 1997. Comparison and Application of Ribosome Spacer DNA Amplicon Polymorphisms and Pulsed-Field Gel Electrophoresis for Differentiation of Methicillin-Resistant Staphylococcus aureus Strains. J. Clin. Microbiol. **35**:881-885.
7. **Jayaratne P., and C. Rutherford.** 1999. Detection of Methicillin-Resistant Staphylococcus aureus (MRSA) from Growth and Manitol Salt Oxacillin Agar Using PCR for Nosocomial Surveillance. Diagn. Microbiol. Infect. Dis. **35**:13-18.

8. **Mitsuda T., K. Arai, M. Ibe, T. Imagawa, N. Tomono, and S. Yokota.** 1999. The Influence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Carriers in a Nursery and Transmission of MRSA to their Households. J. Hosp. Infect. **42**:45-51.

9. **Champers H. F.** 1997. Methicillin Resistance in Staphylococci : Molecular and Biomedical Basis and Clinical Implications. Clin. Microbiol. Rev. **10**: 781-791.

10. **Belkum A. V.** 1994. DNA Fingerprinting of Medically Important Microorganisms by Use of PCR Clin. Microbiol. Rev. 7: 174-184.

11. **Wichehause T. A., S. Kern, V. Schafer, and V. Brade.** 1999. Rapid Detection of Epidemic Strains of Methicillin-Resistant *Staphylococcus aureus*. J. Clin. Micorbiol. **37**:690-693.

12. **Tenover F. C., R. Abbett, G. Archer, J. Biddle, S. Byrne, R. Georing, G. Hancock, G. A. Hebert, B. Hill, R. Hollis, W. R. Jarvis, B. Kereiswirth, W. Eisner, J. Maslow, L. K. McDougal, J. Michael, M. Mulligan, and M. A. Pfaller.** 1994. Comparison of Traditional and Molecular Methods of Typing Isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **32**:407-415.

13. **Blumberg H. M., D. Rimland, J. A. Kiehlbauch, P. M. Terry, and K. Wachsmuth.** 1992. Epidemiologic Typing of Staphylococcus aureus by DNA Restriction Length Polymorphisms of rRNA Genes: Elucidation of the Clonal Nature of a Group of Bacteriophage-Nontypeable, Ciprofloxacin-Resistant, Methicillin-Susceptible *S. aureus* Isolates. J. Clin. Microbiol. **30**: 362-369.

14. **Zee A., H. Verbakel, J. Zon, I. Frenay, A. Belkum, M. Peeters, A. Buiting, and A. Bergmans.** 1999. Molecular Genotyping of *Staphylococcus aureus* Strains: Comparison of Repetitive Element Sequence-Based PCR with Various Typing Methods and Isolation of a Novel Epidemicity Marker. J. Clin. Microbiol. **37**: 342-349.

15. **Berridge B. R. , J. D. Fuller, J. Azavedo, D. E. Low, H. Bercovier, and P. F. Frelier.** 1998. Development of Specific Nested Oligonucleotide PCR Primers for the *Streptococcus iniae* 16S-23S Ribosomal DNA Intergenic Spacer. J. Clin. Microbiol. **36**: 2778-2781.

16. **Sousa M. A. , S. Sanches, M. L. Ferro, M. J. Vaz, Z. Saraiva, T. Tendeiro, J. Serra, and H. de Lencaster.** 1998. Intercontinental Spread of a Multidrug-Resistant Methicillin Resistant *Staphylococcus aureus* Clone. J. Clin. Microbiol. **36**:2590-2596.

17. **Vector J. C., P. J. Carpenter, and N. Wood.** 1997. Methicillin Resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Internet file.

18. **Hoefnagels-Schuermans A., W. E. Peetermans, M. J. Struelens, S. van Lierde, and J. van Eldere.** 1997. Clonal Analysis and Identification of Epidemic Strains of Methicillin-Resistant staphylococcus aureus by Antibiotyping and Determination of Protein A Gene and Coagulase Gene Polymorphisms. J. Clin. Microbiol. **35**: 2514-2520.

19. **Schmitz, F. J., M. Steiert, H. V. Tichy, B. Hofmann, J. Verhoef, H. P. Heinz, K. Kohrer, and M. E. Jones.** 1998. Typing of Methicillin-Resistant *Staphylococcus aureus* isolates from Dusseldorf by Six Genotypic Methods. *J.* Med. Microbiol. **4**:341-351.

20. **Mulligan, M. E., M. Katherine, B. Staphylococcus aureus Ribner, H. C. Harold, J. F. John, J. A. Korvick, C. A. Carol, and V. L. Yu.** 1993. Methicillin-Resistant *Staphylococcus aureus* : A Consensus of the Microbiology, Pathogenesis, and Epidemiology With Implications for Prevention and Management. Am. J. of Med. **94**: 313-328.

21. **Deplano, A., M. Vaneechoutte, G. Verschraegen, and M. J. Struelens.** 1997. Typing of *Staphylococcus aureus* and *Staphylococcus epidermidis* Strains by PCR Analysis of Inter-IS256 Spacer Length Polymorphisms. J. Clin. Microbiol. **35**: 2580-2587.

22. **Cotter, L., M. Lynch, B. Cryan, P. Greer, and S. Fanning.** 1997. Investigation of a Methicillin-Resistant *Staphylococcus aureus* (MRSA) Outbreak in an Irish Hospital: Triplex PCR and DNA Amplification Fingerprinting. J. Hosp. Infect. **36**:37-47.

23. **Olmos, A., J. J. Camarena, J. M. Nogueria, J. C. Navarro, and J Risen.** 1998. Application of An Optimized and Highly Discriminatory Method Based on Arbitrarily Primed PCR for Epidemiologic Analysis of Methicillin-Resistant *Staphylococcus aureus* Nosocomial Infections. J. Clin. Microbiol. **36**:1128-1134.

24. **Tyler, K. D., G. Wang, S. D. Tyler, and W. M. Johnson.** 1997. Factors Affecting Reliability and Reproducibility of Amplification-Based DNA Fingerprinting of Representative Bacterial Pathogens. J. Clin. Microbiol. **35**:339-346.

25. **Boyce, J. M., M. M. Jackson, and G. Pugliese.**1994. Methicillin - Resistant *Staphylococcus aureus* (MRSA): A briefing for Acute Care Hospitals and Nursing Facilities. *Infect.* Control. Hosp. Epidemiol. **15**:105-115.

26. **Wang, J., K. Sawai, K. Tomono, Y. Yanagihara, Y. Hikarata, J. Matsuda, C. Mochida, F. Lori, H. Koga, T. Tashiro, and Staphylococcus aureus Kohno.** 1998. Infections Caused by Multiple Strains of Methicillin-Resistant *Staphylococcus aureus* - A pressing Epidemiological Issue. J. Hosp. Infect. **39** :221:225.

27. **Smeltzer, M. S., A. F. Gilapsy, F. L. Pratt, and M. D. Thames.** 1997. Comparative Evaluation of Use of *can, fnbA, fnbB, and hlb* for Genomic Fingerprinting in the Epidemiological Typing of *Staphylococcus aureus*. J. Clin. Microbiol. **35**: 2444 - 2449.

28. **Hartstein, A. I., C. L. Pheleps, R. Y. Y. Kwok, and M. E. Mulligan.** 1995. In Vivo Stability and Discriminatory Power of Methicillin-Resistant *Staphylococcus aureus* Typing by Restriction Endonuclease Analysis of Plasmid DNA Compared with Those of Other Molecular Methods. J. Clin. Microbiol. **33**: 2022 - 2026.

29. **Mendoza, M., H. Meugnier, M. Bes, J. Etienne, and J. Freney.** 1998. Identification of Staphylococcus Species by 16S-23S rDNA Intergenic Spacer PCR Analysis. Int. J. Syst. Bacteriol. **48**: 1049-1055.

30. **Rudolph, K. M., A. J. Parkinson, and M. C. Roberts.** 1998. Molecular Analysis by Pulsed-Field Gel Electrophoresis and Antibiogram of *Streptococcus pneumoniae* Serotype 6B Isolates from Selected Area within the United States. J. Clin. Microbiol. **36**: 2703-2707.

31. National Committee for Clinical Laboratory Standards.1993. *NCCLS document M2-5A.*, 5th ed., approved standard. National Committee for Clinical Laboratory Standards, Villanova, PA.

32. **Na'was, T., A. Hawwari, E. Hendrix, J. Hebden, R. Edelman, M. Martin, W. Campbell, R. Naso, R. Schwalbe, and I. Fattom.** 1998. Phenotypic and Genotypic Characterization of Nosocomial *Staphylococcus aureus* Isolates from Trauma Patients. J. Clin. Microbiol. **36**: 414-420.

33. **York, M. K., L. Gibbes, F. Chehab, and G. F. Brooks.** 1996. Comparison of PCR Detection of *mecA* with Standard Susceptibility Testing Methods to Determine Methicillin Resistance in Coagulase-Negative Staphylococci. J. Clin. Microbiol. **34**: 249-253.

34. **Humphreys, H., and G. Duckworth.** 1997. Methicillin-Resistant *Staphylococcus aureus* (MRSA) - A reappraisal of Control Measures in the Light of Changing Circumstances. J. Hosp. Infect. **36** : 167-170.

35. **Durmaz, B., R. Durmaz, K. Sahin.** 1997. Methicillin -Resistance Among Turkish Isolates of *Staphylococcus aureus* Strains from Nosocomial and Community Infections and their Resistance Patterns Using Various Antimicrobial Agents. J. Hosp. Infect. **37**: 325-329.

36. **Jorgensen, M., R. Givney, M. Pegler, A. Vickery, and G. Funnell,** 1996. Typing Multidrug Resistant *Staphylococcus aureus* : Conflicting Epidemiological Data Produced by Genotypic and Phenotypic Methods Clarified by Phylogenetic Analysis. J. Clin. Microbiol. **34**: 398-403.

37. **Kluytmans, J., A. Belkum, and H. Verbrugh,** 1997. Nasal Carriage of *Staphylococcus aureus* : Epidemiology, Underlying Mechanisms, and Associated Risks. J. Clin. Microbiol. **10**:505-520.

38. **Van Wamel, W., A. Fluit, T. Wadstorm, H. Dijk, J. Verhoef, and C. Vanderbrouck-Grauls,** 1995. Phenotypic Characterization of Epidemic versus Sporadic Strains of Methicillin-Resistant *Staphylococcus aureus*. J. Clin. Microbiol. **35**:1769-1774.

39. **Kluymans, J., W. Leeuwen, W. Goessens, R. Hollis, Staphylococcus aureus Messer, L. Herwaldt, H. Bruining, M. Heck, J. Rost, N. Leeuwen, A. Belkum, and H. Verbrugh,** 1995. Food-Initiated Outbreak of Methicillin-Resistant *Staphylococcus aureus* Analyzed by Pheno- and Genotyping. J. Clin. Microbiol. **10**:1121-1128.

40. **Villari, P., C. Farullo, I. Torre, and E. Nani,** 1998. Molecular Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a University Hospital in Italy. Eur. J. Epidemiol. **14:** 807-816.

41. **Belkum, A., J. Kluytmans, W. Leeuwen, R. Bax, W. Quint, E. Peters, A. Fluit, C. Vandenbroucke-Grauls, A. Brule, H. Koeleman, W. Melchers, J. Meis, A. Elaichouni, M. Vaneechoutte, F. Moonens, N. Maes, M. Struelens, F. Tenover, and H. Velbrugh,** 1995.

Multicenter Evaluation of Arbitrarily Primed PCR for Typing of *Staphylococcus aureus* Strains. J. Clin. Microbiol. **33**:1537-1547.

42. **Macfarlane, L., J. Walker, R. Borrow, B. A. Oppenheim, and J. Fox,**1999. Improved Recognition of MRSA Case Clusters by the Application of Molecular Subtyping Using Pulsed-Field Gel Electrophoresis. J. Hosp. Infect. **41**:29-37.

43. **Terpstra, S., G. Noordhoek, H. Voessten, B. Hendriks, and J. Degeners,** 1999. Rapid Emergence of Resistant Coagulase-Negative Staphylococci on the Skin after Antibiotic Prophylaxis. J. Hosp. Infect. **43**:195-202.

44. **Kearns, A., P. Seider, J. Wheeler, R. Freeman,, and M. Steward,** 1999. Rapid Detection of Methicillin-Resistant Staphylococci by Multiplex PCR. J. Hosp. Infect. **43**:33-37.

45. **Struelens, M., A. Deplano, C. Godard, N. Maes, and E. Serruys,** 1992. Epidemiological Typing and Delineation of Genetic Relatedness of Methicillin-Resistant *Staphylococcus aureus* by Macrorestriction Analysis of Genomic DNA by Using Pulsed-Field Gel Electrophoresis. J. Clin. Microbiol. **30**:2599-2605.

46. **Johnson, A. P.,** 1998. Antibiotic Resistance among Clinically Important Gram-Positive Bacteria in the UK. J. Hosp. Infect. **40**:17-26.

47. **Bingen, E., M. Barc, N. Brahimi, E. Vilmer, and F. Beaufils,** 1995. Randomly Amplified Polymorhic DNA Analysis Provides Rapid Differentiation of Methicillin-Resistant Coagulase-Negative Staphylococcus Bacteremia Isolates in Pediatric Hospital. J. Clin. Microbiol. **33**:1657-1659.

48. **Mulligan, M. E., R. Kwok, D. Citron, J. John, and P. Smith,** 1998. Immunoblots, Antimicrobial Resistance, and Bacteriophage Typing of Oxacillin-Resistant *Staphylococcus aureus*. J. Clin. Microbiol. **26**:2395-2401.

49. **Geha, D. J., J. Uhl, C. Gustaferro, and D. Persing,** 1994. Multiplex PCR for Identification of Methicillin-Resistant Staphylococci in the Clinical Laboratory. J. Clin. Microbiol. **32**:1768-1772.

50. **Tambic, A., E. G. M. Power, T. Tambic, I. Snur, and G. L. French,** 1999. Epidemiological **Analysis** of Methicillin-Resistant *Staphylococcus aureus* in a Zagreb Trauma Hospital Using a Randomly Amplified Polymorphic DNA-Typing Method. Eur. J. Microbio. Infect. Dis. **18**:335-340.

الملخص باللغة العربية

تشكل البكتيريا المقاومة للمضاد الحيوي الميثيسلين والتي تعرف اختصارا ب (MRSA) مشكلة عالمية كبيرة لما تسببه من أمراض للمرضى أثناء إقامتهم في المستشفيات. من هنا جاءت الحاجة إلى وسائل سريعة وناجحة وعملية للحصول على معلومات دقيقة عن العلاقة بين الأنواع المختلفة من هذه البكتيريا، وذلك من أجل تطبيق إجراءات سريعة وفعالة للسيطرة عليها والحد من انتشارها.

تهدف هذه الدراسة إلى دراسة توزيع ٤٤ عينة بكتيرية من نوع MRSA تم جمعها من ثلاثة مستشفيات في فلسطين في الفترة الواقعة ما بين شهري آذار وأيار من عام١٩٩٨، كما تهدف أيضا إلى فحص قدرة تقنية استخدام المضادات الحيوية (Antibiogram) بالإضافة إلى تقنيتين جينيتين تعرفان اختصارا ب (RS-PCR) و (AP-PCR) على تحديد مدى العلاقة بين هذه العينات.

لقد كانت قدرة هذه التقنيات المختلفة على التفريق بين الأنواع المختلفة من بين هذه العينات كالتالي: تم تحديد ١٧ نوعا من بين هذه العينات وذلك باستخدام ١٥ مضادا حيويا مختلفا، وقد أعطيت هذه الأنواع الأرقام الإنجليزية من 1 وحتى 17 . لقد كانت جميع عينات MRSA مقاومة للمضادين الحيويين أوكسسلين وأمبسلين وحساسة للمضادين الحيويين فانكومايسين وريفامبيسين، فيما كانت هذه العينات مقاومة للمضادات الحيوية الأخرى بنسب متفاوتة. لقد وجد أيضا أن ٧٨% تقريبا من هذه العينات كان مقاوما لأكثر من ٣ مضادات حيوية معا.

لقد أظهر استخدام تقنية RS-PCR ١١ نوعا مختلفا من بين ٤٠ عينة MRSA أعطيت أرقاما لاتينية كبيرة من I وحتى XI في حين لم تتمكن هذه التقنية من تحديد نوع ٤ عينات. أما بتطبيق تقنية AP-PCR وباستخدام BG2 primer ، فقد تم الحصول على ١٥ نوعا مختلفا من بين ٣٨ عينة MRSA أعطيت أحرفا إنجليزية كبيرة من A وحتى O ، في حين لم تستطع هذه التقنية من تحديد نوع ٦ عينات. وبتطبيق نفس التقنية ولكن باستخدام P7 primer فقد تم الحصول على ١٤ نوعا مختلفا من بين ٤٢ عينة MRSA أعطيت أحرفا إنجليزية صغيرة من a وحتى n، فيما كانت هذه التقنية غير قادرة على تحديد نوع عينيتين منها.

لقد كانت قدرة التقنيات الجينية في هذه الدراسة على التمييز بين الأنواع المختلفة ضمن عينات MRSA أكثر من قدرة طريقة فحص المضادات الحيوية على ذلك، كما أنها أعطت دليلا واضحا على أن مصدر انتشار هذه البكتيريا في وحدتي الأطفال والعناية المكثفة في مستشفى رفيديا هو بيئة هاتين الوحدتين وذلك لأن معظم العينات التي تم جمعها من البيئة والأشخاص الحاملين لهذه النوع من البكتيريا من هاتين الوحدتين كانت تشترك بنفس النوع الجيني.

بجمع نتائج التقنيتين الجينيتن RS-PCR و AP-PCR معا نحصل على ٥ مجموعات جينية بينما نحصل على ٦ مجموعات جينية بجمع نتائج تقنية AP-PCR باستخدام BG2 primer و P7 primer. عند دراسة توزيع العينات على هذه المجموعات الجينية ومصدر الحصول عليها من المستشفى، تبين لنا أيضا أن بيئة وحدة الأطفال ووحدة العناية المكثفة هي مصدر انتشار هذه البكتيريا في هاتين الوحدتين.

وبذلك نستطيع القول بأن استخدام أكثر من تقنية جينية وجمع نتائجها معا يمكننا وبدقة من التمييز بين الأنواع المختلفة من البكتيريا وتحديد مصدر انتشارها مما يسهل وضع الخطط والإجراءات للسيطرة على هذه الأنواع والحد من انتشارها بين الأقسام المختلفة في المستشفيات.