THE MOLECULAR TYPING OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) INFECTION ISOLATES AND INVESTIGATION OF MECA GENE

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Sümeyra Özen

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Serdal SAKÇALI
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Fahrettin GÜCİN
Supervisor

Examining Committee Members

Prof. Dr. Fahrettin Gücin ……

Assist. Prof. Dr. Fahri Akbaş ……...

Assoc. Prof. Dr. Barık Salih ……..

Assist. Prof. Dr. İffet İrem Uzonur ……

Assist. Prof. Dr. M. Fatih Abasıyanık ……

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate Institute of Sciences and Engineering.

Assoc. Prof. Dr. Nurullah ARSLAN
Director

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Sumeyra OZEN

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Supervisor: Prof. Dr. Fahrettin GUCIN

Co-Supervisor: Assist. Prof. Dr. Fahri AKBAS

ABSTRACT

Multidrug-resistant gram-positive bacteria are a growing problem in both hospitals and community. Methicillin–resistant *Stapphylococcus aureus* (MRSA) was first reported sporadically in Europe beginning in 1961 and over the span of the last 15 years has emerged as a major multidrug-resistant pathogen worldwide.

Methicilline resistance *Staphylococcus aureus*es are important pathogenic bacteria. Especially they cause serious health problems in the intensive care unit's patients. They can cause death in Turkey and around the world, which patient shave fallen immunity.

Medical treatment is difficult, because of meticillin and the other some antibiotics' resistance, and they must be under the control. Important nosocomial pathogens which from *Staphylococcus aureus*'s adaptation capacity is elevated against environmental conditions such as aridity. Besides, MRSA's are agent bacteria for multipli-antibiotic resistance to the other bacteria. They have caused epidemic. For that reason epidemiologic control is needed.

In this study, have made molecular typing of MRSA isolates which isolated patients from Istanbul University Medicine Faculty by using plasmid profile analysis. MecA gene which is responsible for methicillin resistance was detected with PCR. By comparing to isolated plasmid DNA's and detecting mecA gene, to make contribution to epidemiologic studies are aimed.

Keywords: MRSA, *Staphylococcus aureus*, Antibiotic resistance, Molecular typing, Epidemiology, mecA gene

METİSİLİN REZİSTAN *STAPHYLOCOCCUS AUREUS* (MRSA) İNFEKSİYONLARINDA İZOLATLARIN MOLEKÜLER TİPLENDİRİLMESİ VE MECA GENİNİN ARAŞTIRILMASI

Sümeyra ÖZEN

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Tez Yöneticisi: Prof. Dr. Fahrettin GÜCİN

Eş Tez Yöneticisi: Yard. Doç. Dr. Fahri AKBAŞ

ÖZ

Çoklu dirençli gram-pozitif bakteriler tüm hastaneler ve toplumlarda büyüyen bir problemdir. Metisilin dirençli *Staphylococcus aureus*'un 1961'in başlarında Avrupa'da görülmeğe başlandığı ilk kez rapor edilmiş, sonraki onbeş yıldan günümüze dünya genelinde çoklu antibiyotik direncine sahip patojenler olarak yayılmışlardır.

Metisilin dirençli *Staphylococcus aureus*'lar (MRSA) önemli patojen bakterilerdir. Özellikle yoğun bakım ünitelerindeki hastalarda ciddi sağlık problemlerine yol açmaktadırlar. Türkiye ve dünyada yoğun bakım ünitelerindeki vücut direnci düşmüş hastalarda ölümlere neden olabilmektedirler.

Metisilin gibi güçlü antibiyotiklere bile direnç geliştirdiklerinden dolayı tedavi zorlukları vardır ve kontrol altında tutulmaları gerekir. Önemli nozokomiyal patojenlerden olan *Staphylococcus aureus*'un bulunduğu ortama uyum yeteneği yüksektir. Kuraklık gibi dış ortam koşullarına da dayanıklıdır.

Ayrıca çoklu antibiyotik direncinin diğer bakterilere de aktarılmasında etken ajan bakteriler oldukları düşünülmektedir. Bunlar salgınlara neden olmaktadır. Bu yüzden epidemiyolojik takip gereklidir.

İstanbul Ünivesitesi Cerrahpaşa Tıp Fakültesindeki kliniklerin hastalarından izole edilmiş MRSA izolatlarının plazmid profil analizi ile moleküler tiplendirilmeleri yapıldı. Metisilin direncinden sorumlu olan mecA geni PCR ile saptanmıştır. İzole edilen Plazmid DNA' nın karşılaştırılmaları yapılarak ve mecA geninin saptanması yapılarak epidemiyolojik çalışmalara katkı sağlamak amaçlanmıştır.

Anahtar Kelimeler: MRSA, *Staphylococcus aureus*, Antibiyotik Dirençliliği, Moleküler Tiplendirme, Epidemiyoloji, mecA Geni

This dissertation is dedicated to my parents and my husband.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

IDCU	:	Infectious Disease Health Servise
DNAse	:	Deoxyribonuclease
S.aureus	:	*Staphylococcus aureus*
MRSA	:	Methicillin Resistant *Staphylococcus Aureus*
ONS	:	Office of National Statistics
HA-MRSA	:	Health Associated-Methicillin Resistant *Staphylococcus Aureus*
CA-MRSA	:	Community Associated-Methicillin Resistant *Staphylococcus Aureus*
VISA	:	Vancomycin Intermediate *Staphylococcus Aureus*
VRSA	:	Vancomycin Resistant *Staphylococcus Aureus*
TMP/SMX	:	Trimethoprim/Sulfamethoxazole
MIC	:	Minimal Inhibitory Concentration
PBP	:	Penicillin Binding Protein
MSSA	:	Methicillin Sensitive *Staphylococcus Aureus*
BORSA	:	Boderline Resistant *Staphylococcus Aureus*
MODSA	:	Modified Resistant *Staphylococcus Aureus*
CLSI	:	Clinical and Laboratory Standards Institute
REAP	:	Restriction Endonuclease Analysis of Plasmids
PFGE	:	Pulsed Field Gel Electrophoresis
RE	:	Restriction Enzyme
RFLP	:	Restriction Fragment Length Polymorphisim
IS	:	Insertion Sequences
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplified Polymorphic DNA
REP	:	Repetitive Extragenic Palindromic
ERIC	:	Enterobacterial Repetitive Intergenic Consensus
CFLP	:	Cleaves Fragment Length Polymorphism
AFLP	:	Amplified Fragment Length Polymorphism
PPA	:	Plasmid Profile Analysis

CHAPTER 1

INTRODUCTION

1.1 *STAPHYLOCOCCUS AUREUS*

1.1.1 General Properties

Staphylococci were first examine and cultered by Pasteur and Koch. But, Ogyston and Rosenbach made initial detailed studies on staphylococci in 1881 and 1884. In 1881, Ogyston observed grape-like clusters of bacteria in pus from human abscesses and than he gave its genus name Staphylococcus. Three years later, Rosenbach was able to isolate and grow these bacteries in pure culture. He gave this bacteria specific epithet Staphylococcus aureus because of the yellow-to-orange pigmented appearance on their colonies. Rosenbach demonstrate that *S.aureus* was responsible for wound infections and fronculosis (Fluit and Shmitz, 2003).

Taxonomically, the genus Staphylococcus aueus is in the Bacterial family Micrococcaceae (Araz, 2009). *S.aureus* is non-motile, non-spor forming, catalase positeve, facultative-aerobe with an optimum growth temperature of 37°C but they better proliferate in aerobic ambience.(Waldwogen et. al., 2000 , PR et al., 1999)

The Staphylococcus bacterium commonly found on the skin and mucous membranes of humans and many species of animal (IDCU, 2007). They synthesized lactic asit by fermentation to various hexoses, pentoses, disaccharides and polyol. Mannitol fermentation is distinguishing feature for *S. aureus* .(Bilgehan, 1990)

S. aureus cell wall contains peptidoglycan and teichoic acid. Peptidoglycan is a complex macromolecule which is the essential structure of cell wall. The foundation stone of the structure, are glycan chain. Glycan chain is comprised of disaccharide chains which are formed by alternate alignment of N-acetyl glucosamine and N-acetyl

muramic acid units. On the glycan chain, a tetrapeptide structure bound to lactyl group of muramic acid is present. Cross-links are formed between diamino acid at the third position of tetrapeptide and D-Alanin at fourt position of other tetrapeptide. Structure of those links shall vary among species. Cross links assigns the integrity of cell wall. Cross link level is high in *S. aureus* and this feature ensures the bacteria be resistant against the enzyme lysozyme (Wilkinson, 1997).

Another important component in the cell wall of *S. aureus* is a water-soluble polymer which is comprised of ribitol unit bound with another important component, teichoic acid phosphodiester bounds. Found only in the cell wall of Gram (+) bacteria, this structure provides negative load on cell surface and thus, it play a role in the localization of various metal ions and cations and in the activation of autolytic enzymes (Wilkinson, 1997). Teichoic acids are species-specific antigens of Staphylococci. Ribitol teichoic acid is unique for *S.aureus* (Kingsbury and Wagner, 1992).

The Staphylococcus bacterium is an opportunistic pathogen. It is one of the most common human pathogens whereas in most cases does not result in infection of healthy individuals. With the right circumstances, however, some species of Staphylococcus can result in a variety of soft tissue and blood infections which can sometimes become severe (IDCU, 2007; Mahon and Manuselis, 2000). Since *Staphylococcus aureus* (*S. aureus*) is often leads to serious infections and has proven very efficient at adapting to commonly used antibiotic, it is the most prominent species of Staphylococcus (Fong and Kolia, 2003; Mahon and Manuselis, 2000; IDCU, 2007).

1.1.2 Virulences and Pathogenesis

S. aureus is associated with many virulence factors, including various structural components, exotoxins, and excreted enzymes. A structural component unique to *S. aureus* (but not all strains) is Protein A. Protein A inhibits antibody-mediated clearance in the host by binding to the *Fc* receptors of immunoglobulin. The alpha and beta cytotoxins which are associated with toxins and virulance, they are found in most strains of *S. aureus* that cause membrane damage to a variety of susceptible host cells. Coagulase, again, is unique to *S. aureus* and is an example of a secreted virulence factor with enzymatic activity (which results in clot formation). The clot is thought to serve a protective function for the microorganism, serving as a barrier against macrophage

exposure and phagocytosis (and antibiotic delivery). *S. aureus* strains are also resistant to penicillin. Resistance to antibiotics is propagated in the environment via the exchange of plasmids. Plasmids encoding antibiotic resistance determinants. Betalacamase are the example of the plasmid-encoded enzyme. They inactivates penicillin by cleaving the beta-lactam ring rendering it ineffective (Murray, at al. 2005).

S. aureus are in the first place among the staphylococci which are isolated from clinical samples for infectious agents (Tilton et al., 1987). *S. aureus* cause variety of infections with via toxins and both invasion and systemic spread. Infections caused by *S. aureus* are summarized in Table 1 (Aygen, 1997; Joklik at al., 1992; Waldvogen, 1999).

Table 1.1 Infections caused by *S.aureus*

Toxins cause infections	**Invasion and systemic spread cause infections**
Toxic shock syndrome	Pith tissue infections (eg., mastitis, impetigo)
Lyell's syndrome	Wound infections (eg., cellulite, bacteriemia)
Ptomaine poisoning	Lung and pleura infections (eg., pneumoia)
	Central nervous system infections (eg., meningitis)
	Muscle and skeleton system infections (eg., osteomiyelitis)
	Uriner system infections
	Foreign body infections

Like any infectious organism, the severity of disease is the result of both the pathogen (i.e., respective virulence factors) and host immune responses. As a result, the populations are those with weakened immune systems or poor personal hygiene, such as infants and young children, and hospital patients with intravascular catheters or compromised pulmonary function, are most at risk for *S. aureus* infections (Murray et al., 2005).

HA-MRSA more commonly causes urinary system infections, blood and circulation infections and respiratory tract infections (Eguia and Chambers, 2003).

1.1.3 Identification

Laboratory Diagnostic Methods:

1– Catalase Test: While Micrococcaceae family, including Staphylococci, is catalase-positive test results.

2- Glucose fermentation: Staphylococcus shows the fermentative effect in the medium.

3- Coagulase tests: It is one of most important test for differentiating *S.auerus* from other staphylococci and it is the only pathogenity criterion. It is based on the plasma coagulating characteristic. It is performed using two principal methods.

a- Lam coagulase test: It determines bound coagulase.

b- Tube coagulase test: It determines free coagulase released to the media.

4- Deoxyribonuclease (DNAase): It is one of supportive tests that it forms a transparent layer by dissolving the media the enzyme inoculated (Özgüven 2006).

1.2 METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

1.2.1 Methicillin Resistance

Prior to the introduction of antibiotics, the mortality rates of patients who were infected with *S. aureus* bacteremia were greater than 80% and infectious diseases were the leading cause of death (Lowy, 2003). The discovery of antibiotics by Sir Alexander Fleming in 1928, however, finally gave physicians an effective means of combating *S. aureus* and other bacterial infections (Fong and Kolia, 2003). In 1941, when penicillin was first introduced, it was effective against 90% of clinical *S. aureus* isolates (Murray at al., 2005). However, due to the widespread production and use of this new "wonder drug," resistance to penicillin was observed as soon as two years later. By 1953, 64- 80% of hospital isolates were resistant to penicillin. Over time new antibiotics, including semi-synthetic penicillin antibiotics such as methicillin that were introduced in 1959, contained an *ortho*-dimethoxyphenyl group directly attached to the side chain of the carbonyl group of the penicillin nucleus designed to overcome β -lactamase activity (Yetman and Lynn, 2003).

The development of penicillin resistance in *S. aureus* has led to the manufacture of many synthetic beta-lactam antibiotics, including Methicillin. The overuse and lack of antibiotic regulation has now resulted in the selection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates making infections with these and related strains more difficult to treat. MRSA strains were first isolated in the 1961 and were primarily associated with nosocomial infections, therefore at that time, the risk factors for MRSA infection included long-term hospitalization, surgical procedures, indwelling percutaneus devices, and dialysis (Murray et al., 2005).

MRSA is a significant problem in hospitals worldwide and since 1997 has even started to show signs of decreased susceptibility to vancomycin, often considered the last line of defense (Enright at al., 2002; Lowy 2003). There are two distinct forms of MRSA. The most common form is known as hospital-associated MRSA or HA-MRSA due to its strong association with recent hospitalization. Other common risk factors for HA-MRSA include residence in a longterm care facility, the use of invasive devices, and recent antibiotic use (Beam and Buckley, 2006; Huang at al., 2006). The incidence rates for HA-MRSA are also much higher in the elderly and infants, who account for the majority of deaths resulting from MRSA (ONS, 2008). The second form of MRSA is known as community-associated (CA-MRSA) because it usually affects individuals who have not had recent contact with medical care facilities (Beam and Buckley, 2006; Bootsma, 2006). Some of the major risk factors for CA-MRSA include participation in contact sports, sharing towels or athletic equipment, injection drug use, and living in crowded or unsanitary conditions (Beam and Buckley, 2006; Davis at al., 2007). Children and adolescents are the age groups most often affected by CA-MRSA Huang, 2006; Davis et al., 2007). CA-MRSA also tends to be less multidrug resistant than HA-MRSA and has very distinct molecular features (Stemper et al., 2004; Allen, 2006).

Glycopeptides seems first-line and reliable option in the treatment of MRSA infections. However, treatment-related position of glycopeptides was compromised when Vancomycin Intermediate *Staphylococcus aureus* (VISA) was reported on 1997 in Japan and Vancomycin Resistant *Staphylococcus aureus* (VRSA) was reported on 2002 in U.S. (Özgüven, 2006). To treat methicillin resistant isolates, vancomycin was introduced in the 1950s and resistance to this drug evolved in a stepwise fashion. First there were intermediate vancomycin-resistant *S. aureus* strains (VISA) and by 2002, the

first vancomycin resistant *S.aureus* (VRSA) were isolated (Yetman and Lynn, 2006). Other antibiotics, which are still used successfully to treat some MRSA infections, include trimethoprim/sulfamethoxazole (TMP/SMX), clindamycin, tetracyclins (doxycyclin and minocycline), and the newest drug Linezolid (Maltezou et al., 2006). Novel antibiotics (quinupristin/dalfopristin, daptomycin, and tigecycline) are administered intravenously and research with these agents has shown they look promising in treating serious and/or life threatening MRSA infections (Yetman and Lynn, 2006).

1.2.2 Methicillin Resistance Mechanisms

If minimal inhibitory concentration (MIC) of oxacillin is over 4 μg/ml, methicilline resistance is mentioned. (Ünal, 2004) Mechanisms responsible from the resistance operate in three ways.

1-Chromosamal (intrinsic) methicillin resistance: Most common resistance is formed by acquisition of a new PBP by staphylococci. PBPs are membrane bound enzymes which catalyze terminal cross-linking reaction throughout the integration of peptidoglycan net throughout the synthesis of bacterial cell wall. Their tasks are vital for bacteria. Beta-lactam antibiotics inhibit synthesis of bacterial cell wall by binding to those proteins (Chambers and Sachdevo, 1992). Most common resistance is formed by acquisition of a new PBP by staphylococci. On the contrary to MSSAa, MRSA strains have an additional PBP which is referred as “PBP 2a” (Turnidge and Grayson, 1993; Chambers, 1997). Affinity of PBP 2a to beta-lactam antibiotics is lower than that of other PBPs. PBP 2a is coded by “mecA” gene which is localized in a 2kb DNA segment (Maranan et al., 1997). For occurrence of methicillin resistance in the bacteria, mec A gene should be expressed (Georgopapadakou, 1993). MecA gene is localized between IS431 and IS527 insertional sequence elements on the chromosome. This feature enables the gene shift to other bacteria and to gain genes providing resistance to other drugs (Kloos, 1998).

In chromosomal terms, this resistance provided by said penicillin-binding protein coded by mec A gene and referred as PBP 2a is not solely against methicillin, but it also states against all beta-lactam antibiotics. Such a resistance is either homogenous or heterogeneous (hetero-resistance) (Jacoby and Archer, 1991).

A. Homogenous resistance: All bacteria forming the bacterial colony carry mecA gene and the gene is functional in all bacteria. They cause high-level resistance.

B. Heterogeneous resistance: It is more common type of resistance. Although all members of bacterial population have the mecA gene, resistance occurs in one per 10^6-10^8 bacteria.

2. Borderline methicillin resistance: Overproduction of beta-lactamase is the case for controlling plasmide. As they are borderline resistant, they are referred as Borderline Resistant S.aureus (BORSA). Secretion of beta-lactamase principally casuses penicillin resistance; methicillin is resistant against this enzyme, but in 1984, Tornsberry et al. showed overproduction of beta-lactamase as another mechanism for development of methicillin resistance in *S. aureus* strains (Mcdougal and Thornsberry, 1986). In fact, anti-staphylococcal penicillins are resistant against hydrolytic effect of penicillinase secreted by staphylococci, but it was shown that several strains slowly but significantly destroy methicillin and oxacillin by over-secretion of penicillinase (Ünal, 1999; Mcdougal and Thornsberry, 1986).

3-Intermediate methicillin resistance: Recently, beta-lactamase negative and methicillin resistance *S.aureus* strains without mecA gene were isolated. It is believed that there is a new resistance mechanism in those bacteria other than above mentioned two resistance mechanisms. This mechanism is reduced sensitivity due to modified PBPs in staphylococci. Strains with such resistance are referred as MODSA (modified resistant *S. aureus*). Those strains include PBP1 and PBP2 in the normal structure, but affinity of those PBPs to beta-lactam antibiotics is low. (Tomasz et al., 1989).

Among all above mentioned mechanism, PBP2a synthesis is the most common methicillin resistance mechanism. Other two mechanisms lead to low-level methicillin resistance and they are of no importance for treatment, because treatment with beta-lactam antibiotics is possible. On the contrary, available antibiotics for the treatment of intrinsic MRSA are still glycopeptides. Therefore, it is extremely important that methicillin resistance is correctly determined and strains with low-level resistance are differentiated from intrinsically resistant strains (Karabiber and Karahan, 1995).

1.2.3 Detect of MRSA

Particularly, MRSA strains can easily spread via infected patients or hospital personel who are colonized by those strains and thus, they cause infections with treatment difficulties. Therefore, determination of MRSA strains in clinical samples is of importance (Kuehnert et al., 2006).

Ideal method for diagnosis of MRSA is direct determination of mecA gene or PBP 2a protein. However, those methods cannot be applied in all clinical laboratories. As oxacillin is a more stable antibiotic than methicillin, efforts are made for determining resistance strains using disc diffusion, agar screening and agar dilution methods in accordance with the CLSI (Clinical and Laboratory Standards Institute) recommendations via use of oxacillin in the clinical laboratories (Mongkolrattanothai et al., 2003).

Phenotypic test methods used for determination of MRSA are shown in below table.

Table 1.2 Phenotyping tests for used at detect of MRSA

Method	**Culture**	**Antibiotic**	**Inoculum**	**Tempereture and Incubation**	**Criteria for MRSA**
Disc Diffusion	Müller-Hinton agar	1 oxacillin or 5 µg methicillin disc	Swabla 1×10^8 CFU/ml	35ºC 24 hour	Oxacillin zone < 10 mm, methicillin zone < 9 mm
Agar screening	%4 NaCl Müller-Hinton	6 µg/µl oxacillin in test culture	Direct spot 1×10^8 CFU/ml	35ºC 24 hour	Proliferate in spot part
Broth microdilution	%2 NaCl Müller-Hinton Broth	2 time of oxacillin or methicillin diution	Direct suspension 5×10^8 CFU/ml	35ºC 24 hour	Oxacillin MIK > 4 µg/µl

1.3 EPIDEMIOLOGY OF NOSOCOMIAL MRSA

Today, most important question on staphylococci is the methicillin resistance gradually increasing in every year. MRSA colonization gains particular significance at hospitals (Harbarth and Pittet, 2003). *S.aureus* resistance to antibiotics are growing day after day in the worldwide and it is make infections treatment more complex (Springer et al., 2009) Nosocomial infections are very important cause of mortality and morbidity in health-care settings (Bonten and Bootsma, 2010)

First MRSA epidemics were reported at the beginning of ‘60s from United Kingdom. Early MRSA epidemics could be controlled with aminoglycosides. At the end of ‘70s, occurrence of resistance against gentamycine in *S.aureus* isolates led to search for new antimicrobial treatments to be used in hospital epidemics (Çetinkaya and Ünal, 1996; Gürler, 2003). Later, MRSA strains spread not only at hospitals, but also in the population. In several countries, nosocomial MRSA infections up to 80 % were reported (Gürler, 2003). In a recently published study, the mean rate of MRSA in the nosocomial *S.aureus* infections in 173 hospitals located in Europe was reported as 20.8 % (upper and lower limits were 0 and 69 %, respectively) (MacKenzie et al., 2007). The corresponding rate in U.S. as of 2004 is 60 percent (NNIS, 2004).

In the studies conducted in our country, MRSA infection rates ranging between 50 and 100 % were reported (7, 30, 50-53). MRSA is a more important problem particularly for large-scale hospitals. Rate of methicillin resistance was reported as 14.9 % for small hospitals (<200 beds), 20.3 % for medium size hospitals (200-499 beds) and 38.3 % for large-scale hospitals (>500 beds) (Çetinkaya and Ünal, 1996). MRSA strains are carried into any hospital by colonized patients or healthcare personnel and they cross-contaminate patients via hands of healthcare personnel (Tünger, 2004; Verhoef et al., 2004; Dündar, 2000).

Colonization regions include hands, throat, axilla, face, umbilicus, groin and perineal sites, besides nose. The bacteria follows inter-individual, aerosol or direct contact routes from those regions via skin, mucosal membranes and other objects (Shannin et al., 1999).

Principal risk factors for MRSA colonization and infection are as follows;

a – Long-term hospitalization or history of recurrent hospitalization

b – Long-term and intense antibiotic treatment

c – History of hospitalization in intensive care unit

d – Treatment in burn unit

e – Intravascular catheter or exposure to other invasive procedures

f – Surgical procedures

g – Co-morbidities (diabetes, chronic renal failure etc.)

h – Contact with the patient colonized or infected by MRSA

i – Presence of healthcare personnel in the family

Most important control method for preventing colonization is regular and appropriate hand washing applications. There are local and oral treatment options. Intranasal mupirocin efficiently treats both MSSA and MRSA carriage. However, eradication is short-term and individuals are re-colonized. Moreover, there is a risk for occurrence of resistance. Therefore, it may be only administered to patients with high infection risk (Chambers, 1991).

1.4 MOLECULAR TYPING (GENOTYPING) METHODS

Genotyping of bacteria plasmids or chromosomal DNA has eliminated the negative sides of phenotyping and provided stronger data in epidemiological researches. (Tomasz and Lancastre, 1997) Genotyping is superior to phenotyping in typing, repeatability and distinctive power. When the method repeated with a specific strain has the ability to give the same result, this is defined as repeatability (Green and Stratton, 1996; McGowan and Weinstein, 1998; Arbeit, 1995; Bingen, 1994). Molecular typing methods reveal the genetic differences among the epidemiologically irrelevant isolates. The ground of these methods depends on the differences between microorganisms chromosomal and extra-chromosomal genetic elements (Olive and Bean, 1998; Tenover et al., 1997).

1.4.1 Plasmid Profile Analysis (PPA)

Analysis of bacterial plasmids was the first molecular technique used for the epidemiological investigation of MRSA. This technique consists in the extraction of plasmid DNA and subsequent separation of this DNA by electrophoresis in agarose gels. It is an easily executed and interpreted technique, however it has several limitations, especially inherent to the fact that plasmids are mobile extrachromosomal elements that can be spontaneously lost or readily acquired by bacteria. Consequently, epidemiologically related isolated can display different plasmid profiles. Moreover, many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA. The reproducibility of the generated profiles can be affected by the fact that plasmids exist in different spatial conformations (supercoiled, nicked, and linear), which possess different migration velocities when submitted to agarose gel electrophoresis. Both the reproducibility and discriminatory power of plasmid profile analysis can be substantially enhanced by carrying out enzymatic restriction of the plasmids, as this procedure favours the demonstration of differences in the position and frequency of restriction sites between two non-related plasmids, even though they might share the same molecular mass (Hartstein et al., 1995). The majority of MRSA isolates carry plasmids, but when these are absent the isolates are considered nontypeable. Another limitation is the number of plasmids present in these isolates, usually one or two, which leads to poor discrimination between them (Arbeit, 1999; Weller, 2000). Nontheless, plasmid studies are very effective to evaluate the isolates which are belonging to a outbreak in a partiular place (eg., epidemic in a single hospital or service) and limeted time, in a short time (Arbeit, 1997). Discrimination power of plasmid analysis and repeated availability of plasmids can be increased by analysing the plasmids cut with restriction enzyme and restriction fragments consisting of the number and size. This procces is called restriction enzyme analysis of plazmids (REAP). It is technically simple, less number of equipment needs and relatively fast. (Arbeit, 1997; Back et al., 1993; Arbeit, 1999).

1.4.2 Pulse Field Gel Electrophoresis (PFGE)

As it has a high rate of repeatability, this method has been accepted by many researches as a “golden standard” among molecular methods. In this method, strains

produced in liquid and solid medium are mixed with agarose which has a low melting heat, and put into small moulds. DNA isolation is performed through decomposition with a detergent and enzyme (in situ-lysis) and cut with restriction enzymes. As unimpaired DNA is required in PFGE, the traditional DNA isolation, which could lead to DNA fragmentation is not suitable for this method. Following the lysis procedure, agarose moulds are washed well or dialyzed, thus; contaminants such as proteins and carbohydrates are removed. The big chromosomal DNA is held in agarose gel. The bacterial DNA in agarose is cut with a restriction enzyme (RE) which relatively forms few and big fragments (in situ-digestion). Then the moulds in which cut DNA's are found are placed in suitable holes of the gel, and exposed to electrical current whose direction is changed in specific intervals. Such kind of electrical current makes clear differentiation of 10-800 kilobase of DNA segments. Following electrophoresis, the gel is painted with etidhium bromide and made visible with a band profile belonging to each isolate. These bans profiles are assessed with computer programs and the relationships among the strains are presented. In computerized analysis, a "data bank" can be formed with the collection of the PFGE profiles belonging to the analysed microorganisms. Thus; profiles of strains can be compared with previous data (Olive and Bean, 1999).

The genomic differences of the sub types of the same type causes that, the area where they are cut with restriction enzymes is different. Restriction enzyme model of the whole bacteria genome is formed in this way. When the strains analysed are found to be similar with respect to genotypes, two additional restriction analysis with at least two enzymes are suggested in order to differentiate epidemic and endemic strains clearly. PFGE is also used to examine the plasmid DNA.

The PFGE method developed by Schwartz and Cantor in 1984, is a variation of agarose gel electrophoresis. With this electrophoresis method in which the electrical area is pulsed regularly, it is possible to differentiate between DNA fragments in megabase (from 10 kb to 1,5 mb) which cannot be separated with a normal agarose gel electrophoresis (Arbeit, 1997; Tang et al., 2000; Sambrook et al., 1989). When the staphylococci chromosome that is about 2800 kilobase (kb) gross is cut with *Sma*I enzyme (CCCGGG identification range), there emerges a restriction profile which

consists of 15-20 DNA fragments with a gross between 10 -800 kb (Arbeit, 1997; Weller, 2000).

PFGE has two important limitations: 1) A few long incubation periods are required in order that all tampons and enzymes are sufficiently diffused into agarose blocks. Though today, the procedures needed for *S. aureus* have quite been quite shortened, it requires a demanding 2-4 days. However; the DNA obtained in block stays stabile in +4 °C for long years and this DNA can be easily obtained for other procedures. 2) It requires relatively expensive tools and equipments (Arbeit, 1997). And yet, because of factors such as repeatability and powerful differentiation, PFGE has now become a system widely preferred in many laboratories for the typing of staphylococci (Arbeit, 1997; Olive and Bean, 1999; Bannerman et al., 1995). All staphylococci isolates can be typed with PFGE. As in other other methods based on restriction endonuclease enzyme, the repeatability of band patterns is quite high. Specific artefacts may emerge in every stage of southern blot analysis, the patterns obtained with PFGE can be shown simply by painting the patterns with etidhium bromide. Restriction profiles are relatively simpler and the differences between different strains can often be clearly shown (Arbeit, 1997).

It is very important to obtain high quality DNA in PFGE. Thus; cells in cold tampon must be rapidly processed, bacteria must be kept in ice before being added to melted agarose, and agarose mould must be kept in cold (Maslow et al., 1993).

Various factors must be considered when choosing RE. The first one is the G+C content of bacterial DNA. When DNAs with low G+C content (for example, *S. aureus*) are processed with RE, whose identification area is rich in G+C content (for example *Sma*I), they cannot be cut sufficiently. the second one is that; the enzymes whose identification areas are long, form fewer cutting parts compared to the shorter ones.

PFGE method has different types. The simplest type is the “field-inversion gel electrophoresis”. In this system, electrical current is applied in specific intervals and in two ways as forwards and backwards. The time of the forward current is longer. The commonly used type is “*contour-clamped homogenous electric field*” . In this system, a fixed speed electrical current comes in electrodes which are placed in hexagonal 120 degree angles (Swaminathan and Matar, 1993).

There are various factors influencing DNA fragments' getting separated in agarose. Main of these are; agarose concentration, tampon concentration, heat, pulse time, voltage and total electrophoresis time (Maslow et al., 1993).

In definition of the isolates which are obtained from a secretion and are epidemiologically related, PFGE is known to have a high efficiency and differentiation power (Arbeit, 1997). The power of a typing method used in the differentiation of isolates that are epidemiologically unrelated, can only be revealed with the use of commenting criteria that most suitably shows the limits of repeatability (Hunter, 1990). Previously, two strains were considered to be two different genotypes even when only one restriction part was different between them (Ichiyama et al., 1991; Wei and Grabb, 1992). Tenover and his friends have suggested a system to comment on the results of PFGE (Tenover et al., 1995).

In this system, the relationships among isolates are assessed by looking at the band profiles. Criteria to be used in the interpretation of PFGE profiles are given in Table 1.3.

Table 1.3 Criteria to be used in the interpretation of PFGE profiles (Tenover et al., 1995)

Microbiological Comments	The number of Genetic Differances	Number of bands which show differances	Ephidemiological Relations
Same	0	0	Isolate is part of the epidemic
Close relations	1	2-3	Isolate is close related with epidemics
Probable relations	2	4-6	Isolate is probable related with epidemics
Different	≥3	≥7	Isolate is not related with epidemic

According to these criteria;

a) Isolates possessing the same amount and size of bands are considered to be "indistinguishable",
b) Isolates differentiating up to three parts are considered to be "in close relationship",
c) Isolates showing difference in four-six bands are considered to be "in possible relationship",
d) Isolates showing difference in seven or more bands are considered to be "unrelated".
e) In isolates belonging to a single strain, minor differences may emerge based on a few different genetic events (Tenover et al., 1995).

A loss in restriction area can be possible due to point mutation. In that case, two different parts may combine together to form a larger single part. If a new restriction area is gained, then the opposite of this happens. So, if the difference between two isolates is up to three parts, they are considered to be in close relationship (Arbeit, 1997). In case of a difference in four-six parts, we can talk about two independent genetic events. In that case, two isolates are considered to be in possible relationship with each other. If seven or more parts are different, it means there are three or more genetic events, and isolates are considered to be "unrelated" (Tenover et al., 1995).

In MRSA typing, PFGE has been found to be more effective than other methods (Kreistwirth et al., 1993; Prevost et al., 1991; Saulnier et al., 1993). However; according to some studies, PFGE results of different centres did not show enough compatibility (Zuccarelli et al., 1990; Boyce et al., 1993). Nevertheless, for now, PFGE is the most commonly and efficiently used method in MRSA typing (Arbeit, 1997).

In summary, differentiation power of the method is quite well. On the other hand, this is a time consuming and complex system. Moreover, there still is the standardization problem. Some issues about safety must be given importance when applying the method. It is important to be careful in preventing contamination during the processing of bacteria. Considering that the etidhium bromide used in painting the

gel is a powerful mutagen, one must wear a mask and gloves, and try not to pollute the environment when working (Tang et al., 2000).

1.4.3 Southern Blotting and RFLP Methods

Southern blotting has long been used to determine the genomic orders of various prokaryote and eukaryote organisms. To determine the gens whole chromosomal DNA is cut with a restriction enzyme and the fragments are separated in agarose gel with electrophoresis. Using Southern blotting, fragments are transferred to the nitrocellulose or nylon membrane from the agarose gel. Then, the nucleic acid related to membrane is hybridized with a single and more marked gen and homolog probe. Here the point is to determine the areas where restriction enzymes identify in the specific genetic locus being studied. These areas show changes from one strain to another, and as a result, bands in unlike strains are in different sizes. Thus; to express that locations of restriction enzyme areas in identified genetic regions are polymorphic, the method has been named as "restriction fragment length polymorphism" (RFLP). As only the DNA fragments which have been hybridized with probes become visible in RFLP analysis, the results can be easily analyzed.

RFLP analysis is used to determine the restriction profiles of bacterial chromosome and extra-chromosomal DNA, or viral genome (RNA and DNA). The method has four main steps. These are; the isolation of DNA, DNA'S being cut with RE, electrophoresis of the cut DNA, and finally, the visualization of the DNA fragments in the gel. The most important step of the system is DNA's removal from the cell and becoming purified. Otherwise, enzymes cannot cut DNA, or can only cut partially. Restriction enzymes (RE) are enzymes that most specifically cut DNA in particular places, and generally form fragments of 1.000-20.000 base pairs. *Eco*RI, *Cla*I, *Hind*III ve *Hinf*I can be counted among the most frequently used RE (Persing et al., 1993).

RFLP analysis is used in the detection of the mutations in viral strains, determination of viral epidemics and typing of viral factors. This method requires mostly or partially purified viral nucleic acid (Arens, 1999).

RFLP method is a practical and sensitive method. However, enzyme selection is important. It may not be possible to assess numerous or very close bands. While

assessment is relatively easy when working with a low number of microorganisms, comparison becomes difficult when the number increases. Decreasing the number of the bands compared can be a way to overcome these difficulties. For that purpose, DNA fragments cut with enzymes are developed in gel. Following that, a membrane in the same size is put on the gel. DNA fragments are transferred into the membrane through vacuuming (Southern blotting). The next step is the hybridization step. In hybridization, single strand DNA or RNA probes, which are the complements of one of the chains in DNA, are used. To see hybridization, probes must be marked with radioactive materials (32P) or non-radioactive materials (biotin-digoxigenin). As only the DNA fragments that have been hybridized with probe will be visible at the end, specificity will have increased (Loutit, 1991).

In summary; in addition to ribosomal operons, the application of Southern blotting of various different genetic locus, in *Staphylococcus aureus* typing is common. However, a method specifically more effective from others have not been found. Though southern blotting analysis have been simplified with technical accessories and materials and equipments obtained commercially; it is not widely used as expertise requires special tools and equipments (Arbeit, 1997).

1.4.4 Ribotyping

In this method, ribosomal RNAs specific to kind, type or group are used as probes to determine the variations in DNA orders related to ribosomal operons. Whereas various gens are found in a single copy in bacteria, ribosomal operon gens have various copies. This property makes the method simple. In ribotyping, the DNA of strains are isolated, cut with restriction enzymes and after DNA fragments are separated through electrophoresis, they are hybridized with selected probe (rRNA). When ribosomal gen models are found relatively stable within a kind, the power of the method used in separating isolates from each other gets weakened (Green and Stratton, 1996; McGowan and Weinstein, 1998; Podshun and Ulmann, 1998).

Ribosomal operons are formed by nucleotide ranges coding 16S rRNA, 23S rRNA and one or more tRNA additionally. As all staphylococci carry numerous (five-eight) ribosomal operons, they can be typed with ribotyping (Arbeit 1997, Weller, 2000). Ribotypes are quite stable biologically, and they for repeatable patterns. Performance

properties of ribotyping and other Southern blot analysis are directly influenced by the stringency of hybridization circumstances, and the selection of restriction enzyme and probe. In time-consuming and technically complex ribotyping, the criteria about the interpretation of minor band differences have not been standardized yet (Weller, 2000). All isolates isolated in an epidemic most usually have the same ribotype. All the same; unrelated strains are usually able to show the same pattern with one or two different bands, and isolates epidemiologically related are able to show similar minimal changes, the differentiation power and technical usage of this method are limited. According to a study, isolates which have been divided into six ribotypes with ribotyping have been divided into 26 pulsetypes with PFGE (Prevost et al., 1991). The differentiation power of ribotyping is obviously less than PFGE (Arbeit 1997; Tenover et al., 1994).

"Insertion Sequences (IS)" and transposons are also used as probes in typing of staphylococci isolates (Arbeit, 1997; Weller, 2000). Though some IS elements such as IS*431*, IS*256*, IS*1181* have been found in MRSAs, a single element having enough differentiation power to be commonly used in typing of all strains, have not been found. (Arbeit, 1997; Tenover et al., 1994; Weller, 2000) There are also some publications which suggest that PFGE and IS typing should be used together (Mulligan and Arbeit, 1991; Yoshida et al., 1997). Tn*554* is a transposon with a spectinomisin resistance, and it is found in more than 90% of MRSAs (Kreiswirth et al., 1993). Typing can also be made by comparing the bands that have been formed following the cut of Tn*554* with *Cla*I (Weller, 2000). However; the potential complication of this typing method which is based on movable genetic elements is that, apart from bacterial chromosomes, these movable genetic elements can also be found on plasmids (Arbeit, 1997).

Though ribotyping may seem to be advantageous due to its stability and differentiation power; the DNA isolation, cutting with enzymes, electrophoresis, blotting and hybridization steps of the method are time consuming. Furthermore; suitable enzymes must be selected for each kind and differentiation power must be improved through the use of two or more enzymes (Yağcı and Durmaz, 2001).

1.4.5 Polymerase Chain Reaction (PCR) Methods

When three simple reactions (denaturation, combining, DNA synthesis), which causes that, the single chain of 50-2000 base paired DNA or RNA are multiplied more

than one million times within a few hours in a semi automatic system, are repeated in a cycle; this is called as PCR. That it is a fast method which has high sensitivity and specificity, and it can type microorganisms and products directly, are the useful properties of it. On the other hand, it also has some negative properties. It may give faulty positive result which is bound to contaminant DNA's amplification, or the identification of very similar nucleic acid orders of microorganisms that are not related to epidemics; and primers only identify the targeted nucleic acid order. These all decrease the reliability of PCR in epidemiological researches (Green and Stratton, 1996; McGowan and Weinstein, 1998).

Chromosomal DNA's direct cut-off with RE gives a good result in the presence of numerous and all clean DNAs. However in these methods, the hybridization stage which is included to increase specificity prolongs the time. Thus; amplification of the nucleic acid in the sample is conducted, and sub-typing can be performed in a shorter time and with high reliability (Yağcı and Durmaz, 2001).

As PFGE, the method accepted as a standard in typing of staphylococci requires a long time and technical opportunities, alternative methods have been sought. The most important of these are the methods based on "Polymerase Chain Reaction (PCR)". The main property of the PCR method is the fast amplification of a particular DNA range. With this method, it is possible to easily type nearly 50 isolates within 48 hours (Arbeit, 1997; Weller, 2000; Struelens et al., 1993).

PCR method is a practical method commonly used in the identification of infectious diseases. However; the analysis of a single locus is not sufficient for epidemiological differentiation. For example; the detection of mecA gen may give an idea about methicillin resistance, but it is not sufficient to reveal the relationship among strains. Information more than the presence or absence of target ranges is needed in typing of strains. So, different PCR modifications are used (Arbeit, 1997).

In order to eliminate the disadvantages of conventional PCR, new molecular identification methods that are based on the same ground, have been developed. Here are the most frequently used PCR modifications used in MRSA typing:

A. PCR-based locus- spesific RFLP

In this method, specific gen area is amplified regularly through PCR, and is studied through RFLP analysis. And the differences among the strains studied or the antimicrobial resistance is revealed (Olive and Bean, 1999).

B. Random Amplified Polymorphic DNA (RAPD)

The principle of this method, which is also called as arbitrary primed PCR, is the use of 9-10 base ordered random primers that are hybridized with sufficient affinity to chromosomal DNA orders in low combining temperatures. These random primer areas show differences in location and numbers in different strains of a bacteria type. At the end, characteristic bands emerge for each different strain in agarose gel with electrophoresis (Green and Stratton, 1996; McGowan and Weinstein 1998; Olive and Bean, 1999).

C. Rep-PCR

This is fingerprinting of the DNA of bacteria of the DNA elements repeated in bacterial genome, through the study of models specific to strains obtained by amplification through PCR. Two main sets of repeated elements are used for typing. These are: 1) "Repetitive extragenic palindromic (REP) elements"and 2) "Enterobacterial repetitive intergenic consensus (ERIC) elements" (Olive and Bean, 1999).

In recent years, two more molecular typing method has started to be used. These are; the "cleavase fragment length polymorphism (CFLP)" method in which a termostable endonuclease named as "cleavase I" is used, and the fingerprinting technique "amplified fragment length polymorphism (AFLP)" which depends on the selective amplification of DNA fragments obtained by restriction enzymes (Olive and Bean, 1999).

1.4.6 DNA sequence analysis

All molecular methods used in the determination of sub typing depend on the detection of the differences in a DNA range. Double-chained DNA is denaturized in

order to be single-chained, and then, measurement is made to determine the rate of combination to marked complementary single-chained DNA probe (Green and Stratton, 1996; McGowan and Weinstein 1998; Olive and Bean, 1999).

1.5 COMPARISON OF TYPING METHODS

Tenover and his friends have determined criteria about the selection and interpretation of the molecular methods to be used in epidemiological researches. As well as the differentiation power, a superior repeatability, consideration of the complications in implementation and evaluation of results, the cost and the capacity of the laboratory to apply the method are also important in deciding upon the method. For example; DNA sequence analysis is the most difficult with respect to applicability; compared to other methods, DNA sequence analysis and CFLP are more difficult with respect to the interpretation of results; differentiation power is higher in methods other than RFLP and CFLP; the time needed for getting the result of the experiment is longer in PFGE (3-5 days) compared to other methods; repeatability among laboratories is weaker in Rep-PCR, RAPD ve CFLP compared to other methods; considering the cost of the experiment, AFLP and DNA sequence analysis and considering the cost per experiment, CFLP and DNA sequence analysis are the most expensive methods (Tenover et al., 1997).

As a single method to meet all these criteria is not available, it is like a rule to use a combination of systems (Tenover et al., 1994; Shimizu et al., 2000). If strains isolated from a short-term epidemic regarding a single area, are going to be differentiated, one typing method is sufficient. However; in comprehensive epidemiological studies to be performed all over the country, a few typing method must be used together (Tompkins, 1998). Initially, the presence or absence of clonal relationship is tested through fast scanning systems (plasmid profile analysis, PCR-based methods, etc.). If the differentiation power of the fast method used, is not sufficient, confirmation can be made with a reliable method. Today, the best method to meet this need seems to be PFGE (Sloos et al., 2000; Struelens, 1999; De La Puente-Redondo et al., 2000).

CHAPTER 2

MATERIALS & METHODS

2.1 MRSA ISOLATES

In Ocober 2004 – November 2005, have made molecular typing of MRSA isolates which isolated patients from, Istanbul University Cerrahpaşa Medicine Faculty.

Table 2.1 Characteristics of MRSA isolates

Isolates number	Clinic	Sample
1	Intensive care	Tracheal aspirate
2	Gynecology	Urine
4	Internal disease	Cathater
5	Intensive care	Pus
6	Internal disease	Tracheal aspirate
7	General surgery	Tracheal aspirate
8	Emergency surgery	Tissue
9	Intensive care	Tissue
10	Pulmonary Disease	Cathater
11	Internal disease	Bronchovesicular lavage
12	General surgery	Blood
13	Intensive care of surgery	Drain
14	Intensive care of surgery	Endotracheal aspiration
15	Pulmonary surgery	Blood
16	Internal disease	Blood
17	Intensive care of surgery	Urine
18	Neurosurgery	Blood
19	Intensive care of neurosurgery	Aspiration sample
20	Internal disease	Blood
21	Cardiology	Blood
22	Plastic surgery	Blood
23	Neurology	Tracheal aspirate
24	Emergency	Blood
25	Intensive care	Tracheal aspirate

Table 2.1 Characteristics of MRSA isolates

Isolates number	Clinic	Sample
26	General surgery	Intravenous catheter
27	Orthopedy	Material of operation
28	Neurosurgery	Tracheal aspirate
29	Intensive care	Tracheal aspirate
30	Infant	Blood
31	Pulmonary disease	Tracheal aspirate
32	Pulmonar disease	Tracheal aspirate
33	Neurology	Tracheal aspirate
34	Hematology	Blood
35	Hematology	Blood
36	Hematology	Blood
37	Hematology	Blood
38	Hematology	Blood
39	Hematology	Blood
40	Hematology	Blood
41	Hematology	Blood
42	Hematology	Blood
43	Hematology	Blood
44	Hematology	Blood
45	Hematology	Blood
46	Hematology	Blood
47	Hematology	Blood
48	Hematology	Blood
49	Hematology	Blood
50	Hematology	Blood
51	Hematology	Blood
52	Hematology	Blood

2.2 EXPERIMENTAL PROCESSES

2.2.1 Preparation of MRSA Isolates

To growth the bacterial colonies we used blood agar. For the preparation of 800ml blood agar we got 32 g agar dissolved in 800 mL distilled water. The media were autoclaved at 121^{o}C for 20 minutes, then under steril conditions poured the blood without to froth on the media. Afterwards, the sterile media were poured into sterile Petri dishes. In the next stage we used Luria – Bertani (LB) broth (Merck) for the

cultivation of *Staphylococcus aureus.* For the preparation of 800 mL LB agar medium, 16 g powder was suspended in 500 mL of disstilled water. The suspention was heated to boiling while stirring to dissolve all ingredients completely. The media were autoclaved at 121^{o}C for 20 minutes, then cooled to 50^{o}C. Then e would select a single colony from the petri dishes. We take the single colonies with pippetes and have put in steril Falcon tubes which are containing 3 mL LB broth. *S. aureus* was grown on LB agar at 25°C . For aerobic growth, Falcon tubes were shaken continuously on a rotary shaker (Sartorious Certomat IS) at 150 rpm and 25°C. After 24 h of growth, the suspention were pured into a sterile 1.5 mL Ependorf tubes and centrifuged at 5,000 rpm, for 2 min at 4°C. After centrifugation, the supernatant was discarded and ependorf tubes stored at at -70°C. The remaining petri dishes, LB broth and Blood agar stored at 4°C.

2.2.2 Typing Methods

2.2.2.1 Plasmid Isolation

Before the plasmid isolation, 10 µl lysostaphin (1200U/ml) and 20 µl lysozyme (5mg/ml) was added aseptically to sample which was previously stored at -70^{o}C in the 1.5 ml microcentrifuge tube was incubated at 37^{o}C for 30 min in order to lyse the cells. For plasmid isolation we used High Pure Plasmid Isılation kit (Roche). First of all, 250 µl Suspension Buffer was added to lysed cells and slightly mixed the contents of the tube immediately. Afterwards, 250 µl Lysis Buffer was added and slowly turned upsed down 5-6 times. The tube was incubated for 5 minutes at room temprerature. For protein and lipids degradation in the environment we added 350 µl chilled Binding Buffer and mixed gently. The tube was incubated 5 minutes on ice. Then tube was centrifuged for 15 minutes at 13000 x g. Pelet was discarded and we transfered the supernatant to High Pure fitler tube. The tube centrifuged at 13000 x g for 1 minute. The flowthrough was discarded. 700 µl Washing Buffer II was added. The fitler tube centrifuged at 13000 x g for 1 minute. Flowthrough was discarded. The Filter Tube-Collection Tube assembly was spinned for 10 s at maximum speed (approx. 13,000 x g) to remove residual Wash Buffer. The Collection Tube was discarded and the Filter Tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube. 100 µl Elution Buffer was added to the Filter Tube. The tube assembly was centrifuged for 1 min at 13,000 x g. Plasmid DNA ws purifed.

2.2.2.2 Plasmid Profile Analysis

For analysing the plasmids we cut the plasmids with restriction enzyme and then we visualised them in agarose gel electrophoresis. We used SmaI restriction enzyme for cutting plasmids. It is important that the reactions be set up on ice and that the restriction enzyme is added last. When using restriction enzymes, always take the enzyme from the freezer and place immediately on ice. Use a separate pipet tip every time the enzyme is dispensed, to guard against contamination of the enzyme stock. We prepared mix and its were shared 10 µl to the tubes. Finally we added 30 µl sample DNAs to the tubes.

The following was added to a 1.5 mL Eppendorf tube on ice :

10X Restriction Buffer	4 µl × number of sample
Sma I (10u/µl)	0,2 µl × number of sample
Sterile ddH_2O	5,8 µl × number of sample
Final Volume	**10 µl × number of sample**

The suspention was mixed by pipetting and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. Tubes were centrifuged at 5,000 rpm for 1 min. The tubes was incubated over night at 37°C. Afterwards, 1% agarose gel was prepared to visualize the plasmid profiles. 0,5 g powdered agarose (AppliChem, Germany) was dissolved in 50 ml 1 x Tris Acetate EDTA (TAE) buffer by using microwave oven. It was heated on nearly 45 second until boiling by pulse and stirred with 15 second intervals. The gel was cooled to 50°C and 5 of ethidium bromide (5 mg/ml) was added. The gel was then poured into horizontal agarose gel platform and and a comb was placed in the gel before polymerization. 1 µl DNA size marker from Fermentas (GeneRuler^{TM} 1kb DNA Ladder) with 2 µl of Loading Dye was loaded into the well. Then, Products which were already incubated at 37°C were mixed with Loading Dye (Fermantes) and loaded to the wells. The electrophoresis tank was filled with 1X TAE running buffer and the gel was run for 45 minutes at 90 volts. After

running, the gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the bands were visualised and photographed on a UV transilluminator.

2.2.2.3 DNA Isolation

Before the dna extraxtion, 10 µl lysostaphin (12000U/mg) and 20 µl lysozyme (5mg/ml) was added aseptically to sample which was previously stored at -70^{o}C in the 1.5 ml microcentrifuge tube was incubated at 37^{o}C for 30 min in order to lyse the cells. DNA was extracted using a High Pure PCR Template Preparation kit (Roche). Firstly, we completed the samples to 200 µl with disstilled water. Then, 200 µl Binding Buffer and 40 µl reconstituted Proteinase K solution were added to lysed cells and mixed the contents of the tube immediately. The tube was incubated for 10 min at 70^{o}C. After the incubation, sample was mixed with 100 µl isopropanol. One High Pure Fitler Tube was inserted into one Collection Tube. The entire sample was pipetted into upper buffer reservoir of the Fitler Tube. The entire High Pure Tube assembly a standart tabletop microcentrifuge, then the tube assembly was centrifuged for 1 min at 8000 x g. After centrifugation, the Filter Tube was removed from the Collection Tube and the liquid and the Collection tube were discarded. 500 µl Inhibitor Removal Buffer was added to the upper reservoir of the Filter Tube assembly. The centrifugation step was repeated (1min at 8000 x g) and the liquid and Collection Tube were discarded. The Filter Tube was reinserted in a new Collection Tube. 500 µl Wash Buffer was added to the upper reservoir of the Filter Tube. The centrifugation was repeated again. After the centrifugation, the Filter Tube was removed from the Collection Tube and the liquid and the Collection tube were discarded again. The wash step and centrifugation were repeated. Flowthrough was discarded. The Filter Tube-Collection Tube assembly was spinned for 10 s at maximum speed (approx. 13,000 x g) to remove residual Wash Buffer. The Collection Tube was discarded and the Filter Tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube. 200 µl of prewarmed (70^{o}C) Elution Buffer was added to the Filter Tube. The tube assembly was centrifuged for 1 min at 8000 x g.

2.2.2.4 PCR

Thermocycler Techne was used for amplification reactions. PCRs were performed in reaction mixtures of 25 µl containing approximately 0.5 µl of genomic DNA, 2.5 µl 10 X taq buffer (Takara), 2 µl 0,2 µM dNTP, 1.5 µl $MgCl_2$ (10 µM) , 2 µl (10 µM) Forward mecA F 5' AACAGGTGAATTATTAGCACTTGTAAG 3'and Revers mecA R 5' ATTGCTGTTAATATTTTTTGAGTTGAA 3' primers, 0.2 µl taq polimeraz, 14.3 µl dH_2O. PCR conditions were as follows: 1 cycle of 95ºC for 2 min for initial denaturation followed by 30 cycles at 95ºC for 30 s (denaturation), 63ºC for 30 s (annealing) and 72ºC for 30 sec (extension) and 72ºC for 5 min for final extension and storage at 4ºC.

95°C,	2 min	for initial denaturation	
95°C,	30 sec	for denaturation	x 30 cycle
63°C,	30 sec	for annealing	
72°C,	30 sec	for extension	
72°C,	5 min	for final extension	

2.2.2.5 Agarose Gel Electrophoresis

2 % agarose gel was prepared to visualize the PCR products. Gel is prepared adding 1 gram of powered agarose (AppliChem, Germany) gel into 50 ml of 1 x Tris Acetate EDTA (TAE) (Trizma Base, Glacial Acetic Acid, 0.5 M of EDTA (pH 8.0)) buffer (solution and it is boiled until the agarose is completely dissolved in the buffer solution) by using microwave oven. 5 µl of Ethidium Bromide (5 mg/ml) was added when the boiled solutions began to cool down and reach approximately 55 °C. Solution is mixed homogenously by making hand-shaking. It directly poured into 13 x 14x 0.5 horizontal agarose gel platform and including 20 wells is placed. and a comb was placed in the gel before polymerization. Afterwards, 1 µl DNA size marker from Fermentas (GeneRuler^{TM} 100bp DNA Ladder) with 2 µl of Loading Dye mixture were loaded into the first well and 6 µl of ddH_2O mixture were loaded into the other well. Then, totally 25 µl of PCR product with approximately 2 µl of 6x DNA Loading Dye (Fermentas)

were mixed and loaded on to the other wells. The electrophoresis tank was filled with 1X TAE running buffer and the gel was run for 45 minutes at 100 volts. After running, the gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the band was visualised and photographed on a UV transilluminator.

CHAPTER 3

RESULTS

3.1 RESULTS OF PLASMID PROFILE ANALYSIS

8 different types of plasmid profiles were formed at the end of the plasmid profile analysis of *S. aureus* isolates (51), and 7 different size of plasmid bands were observed (1000, 1500, 2300, 2500, 3500,12000, 14000, 15000). In 1 of the analysed isolates 4 plasmid band, in 5 of them 3 plasmid bands, in 28 of them 2 plasmid bands and in 2 of them 1 plasmid bands were observed. No plasmid band was observed in 15 of them. The gel view belonging to plasmid patterns are shown in Picture 3.1, 3.2, 3.3 and 3.4.

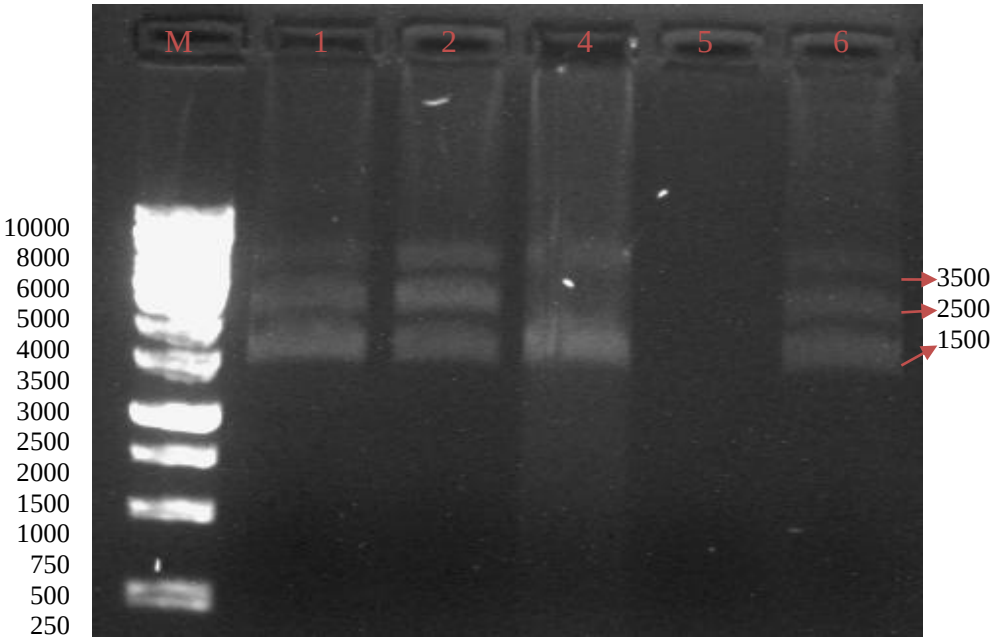

Figure 1.1 Gel image of plasmids number of 1, 2, 4, 5, 6 cut with SmaI enzyme. 1% Agarose gel, M: 1kb DNA Ladder.

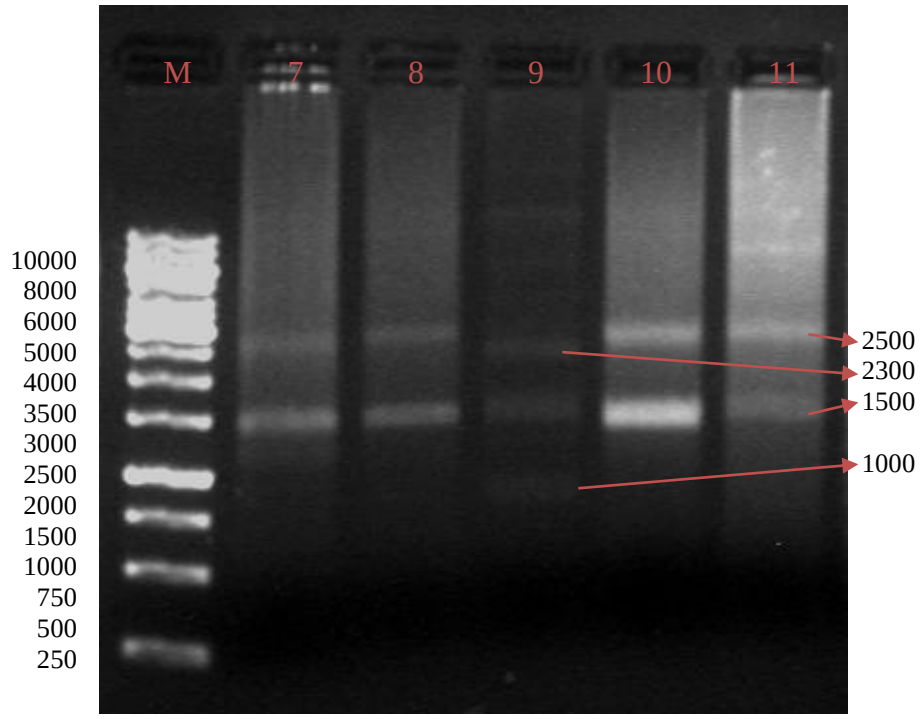

Figure 3.2 Gel image of plasmids number of 7, 8, 9, 10, 11 cut with SmaI enzyme. 1% Agarose gel, M: 1kb DNA Ladder.

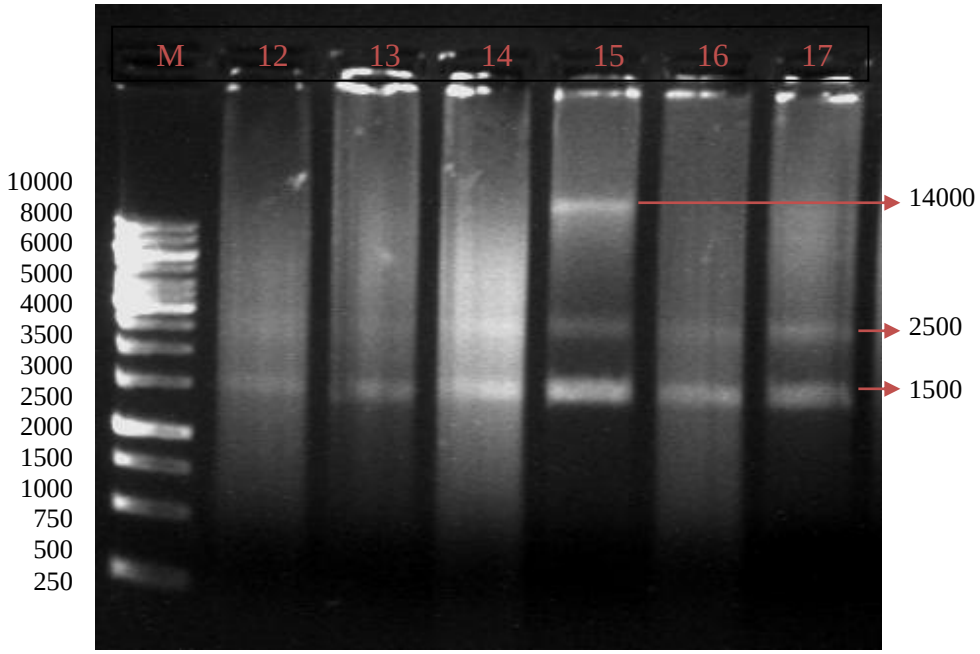

Figure 3.3 Gel image of plasmids number of 12, 13, 14, 15, 16, 17 cut with SmaI enzyme. 1% Agarose gel, M: 1kb DNA Ladder. Bands sizes were given approximately.

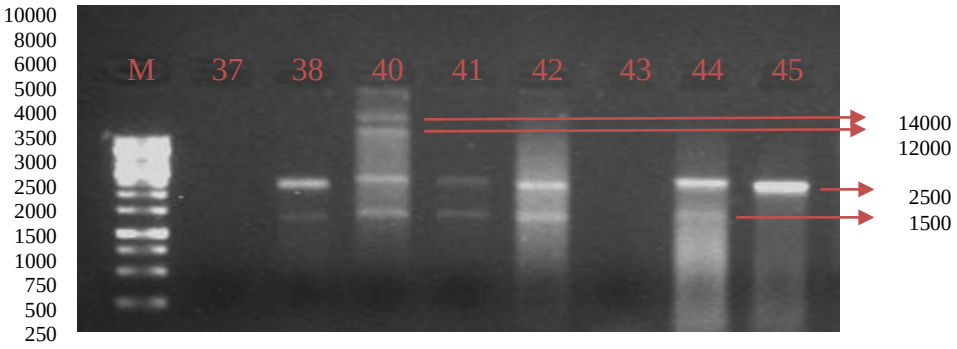

Figure 3.4 Gel image of plasmids number of 37, 38, 40, 41, 42, 43, 44, 45 cut with SmaI enzyme. 1% Agarose gel, M: 1kb DNA Ladder. Bands sizes were given approximately.

Plasmid patterns were represented as P1 (Patern 1), P2,..., P8. Plasmid patterns of the isolates are given in Table 3. 1.

Table 3.1 The number of plasmid bands, molecular weight and patterns of *S.aureus* srains (n=51)

İsolate number	Pattern	Plasmid bands	Size
1	P3	3	1500, 2500, 3500
2	P3	3	1500, 2500, 3500
4	P4	2	1500, 3500
5	P5	0	-
6	P3	3	1500, 2500, 3500
7	P1	2	1500, 2500
8	P1	2	1500, 2500
9	P7	3	1000, 1500, 2300
10	P1	2	1500, 2500
11	P1	2	1500, 2500
12	P1	2	1500, 2500
13	P1	2	1500, 2500
14	P1	2	1500, 2500

Table 3.1 The number of plasmid bands, molecular weight and patterns of *S.aureus* srains (n=51)

İsolate number	Pattern	Plasmid bands	Size
15	P2	3	1500, 2500, 14000
16	P1	2	1500, 2500
17	P1	2	1500, 2500
18	P1	2	1500, 2500
19	P1	2	1500, 2500
20	P5	0	-
21	P1	2	1500, 2500
22	P5	0	-
23	P1	2	1500, 2500
24	P5	0	-
25	P1	2	1500, 2500
26	P5	0	-
27	P1	2	1500, 2500
29	P1	2	1500, 2500
30	P1	2	1500, 2500
31	P1	2	1500, 2500
32	P1	2	1500, 2500
33	P5	0	-
34	P1	2	1500, 2500
35	P5	0	-
36	P5	0	-
37	P5	0	-
38	P1	2	1500, 2500
39	P1	2	1500, 2500
40	P8	4	1500, 2500, 12000, 14000
41	P1	2	1500, 2500
42	P1	2	1500, 2500
43	P5	0	-
44	P1	2	1500, 2500
45	P6	1	2500
46	P5	0	-
47	P5	0	-
48	P5	0	-
49	P1	2	1500, 2500
50	P5	0	-
51	P5	0	-
52	P6	1	2500

3.2 RESULTS OF PCR

S.aureus strains (51) were examined by PCR in terms of the presence of the mecA gene and band size of 150 bp was obtained in 49 of the 51 isolates. (Figure 3.5, Figure 3.6)

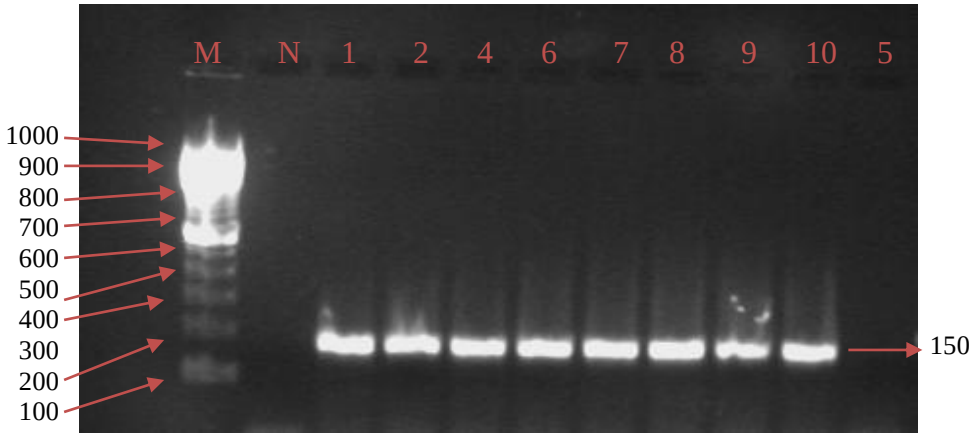

Figure 3.5 PCR detection of the mecA gene for isolates number of 1, 2, 4, 5, 6, 7, 8, 9, 10. 2% agarose gel, M: 100bp Dna Ladder, N: Negative control.

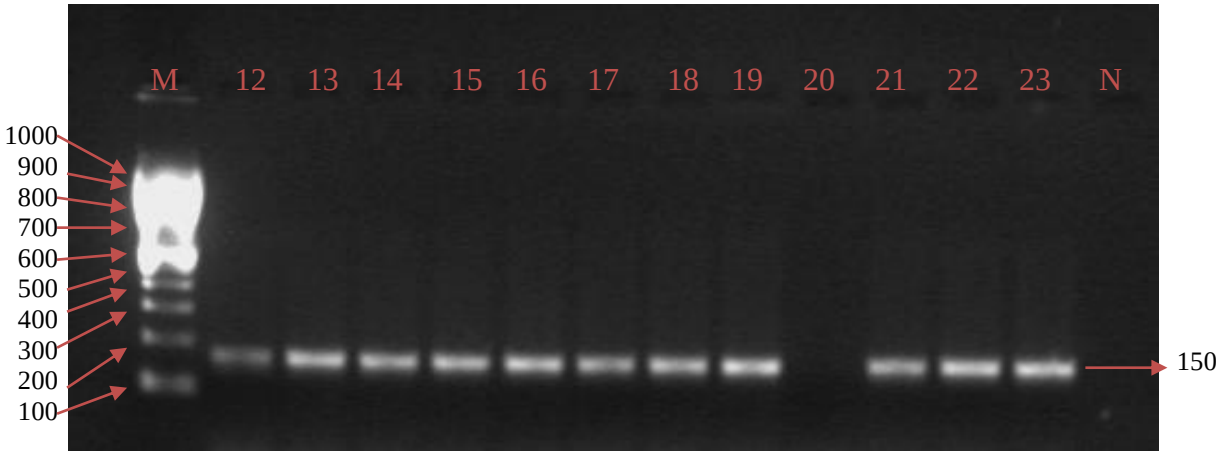

Figure 3.6 PCR detection of the mecA gene for isolates number of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23. 2% agarose gel, M: 100bp Dna Ladder, N: Negative control.

CHAPTER 4

DİSCUSSİON

For more than a hundred years, staphylococci are known to be an important factor of infection. They play role in skin and soft tissue infections, septic arthritis, osteomiyelitis, infectious endocarditis, bacteraemia and prosthetic device infections. In recent years, staphylococci have started to play a prominent role in nosocomial infections, as well. Especially the nosocomial epidemics caused by methicillin resistant *S.aureus* (MRSA) strains have become a serious health problem all around the world due to the cost of infection control measures and the antibiotics used in treatment (Gorwitz et al., 2006; Goto et al., 1999; Hollyoak and Cunn, 1995).

S.aureus is the first bacteria which has developed resistance against penicillin. As well as other antibiotics, this bacteria has even neutralized the methicillin against which many bacteria were unable to develop a resistance. Today, MRSA infections can only be treated by glicopeptide antibiotics. As well as being expensive, these antibiotics are also harmful for human cells. All the same; *S.aureus* strains which were resistant to glycopeptides which had been considered as being insuperable and used as the last shots against MRSAs, started to be isolated in Japan, America, France and England respectively starting from May, 1998. The use of glicopeptide antibiotics in parallel with the increase in MRSA will inevitably lead to the increase in the amount of glicopeptide resistant MRSA strains. At this point, it is very important to control the increase in the amount MRSA strains. The data to be gathered at the end of typing studies will be quite useful in understanding and controlling the spread of the disease in hospitals and among public (Durmaz, 2001).

Single typing is often not accepted in epidemiological researches, and many researches are in favour of using minimum two different methods in epidemiological

researched (Cookson et al., 2007; Sener et al., 2004). Typing methods must have the following properties; high differentiation power, typing ability, good repeatability, applicability in all organisms, practical application, low cost and high mobility (Cookson et al., 2007).

Today, typing methods used in the analysis of hospital based infection epidemics depend on the phenotypic and genotypic differences among MRSA strains (Fung et al., 2001). Molecular typing methods have provided many conveniences for the detection and monitoring of epidemics. Thus; the spread of MRSA clones within and among hospitals can be detected (Roberts et al, 1998).

In this study, have made molecular typing of MRSA isolates which isolated patients from Istanbul University Cerrahpasa Medicine Faculty. Faculty by using plasmid profile analysis and. MecA gene which is responsible for methicillin resistance was detected with PCR.

Analysis of bacterial plasmids was the first molecular technique used for the epidemiological investigation of MRSA. This technique consists in the extraction of plasmid DNA and subsequent separation of this DNA by electrophoresis in agarose gels. It is an easily executed and interpreted technique, however it has several limitations, especially inherent to the fact that plasmids are mobile extrachromosomal elements that can be spontaneously lost or readily acquired by bacteria. Consequently, epidemiologically related isolated can display different plasmid profiles. Moreover, many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA. The reproducibility of the generated profiles can be affected by the fact that plasmids exist in different spatial conformations (supercoiled, nicked, and linear), which possess different migration velocities when submitted to agarose gel electrophoresis. Both the reproducibility and discriminatory power of plasmid profile analysis can be substantially enhanced by carrying out enzymatic restriction of the plasmids, as this procedure favours the demonstration of differences in the position and frequency of restriction sites between two non-related plasmids, even though they might share the same molecular mass (Hartstein et al., 1995).

In some studies carried out (Baumgartner et. al, 1984, Lange et.al, 1999, Aslantaş et.al, 2005), the isolates that contained plasmids were found to be resistant to various antibiotics. The antibiotic resistance in *S. aureus* isolates were emphasized to be coded by the genes of resistance found on plasmids (Lyon and Skurray 1987). Lange et. al, (1999) found that, an isolate containing 5 plasmids was sensitive to all antibiotics studied, one of the three plasmids which did not contain plasmids was resistant to canamycine, and two of them were resistant to penicillin G/ampicillin. Yet, they also found that; when all isolates were studies, the ones not containing plasmids were sensitive to all the antibiotics studied. They emphasized that; the antibiotic resistance genes among *S. aureus* isolates were related to plasmid. All plasmids containing plasmids similarly or not were found to be resistant to antibiotics, as well. However, that most of the resistant isolates contained plasmids supports the claim that, in *S. aureus* isolates, there is a relationship between antibiotic resistance and plasmids.

In our study, 70,6% of the strains for which plasmid studies were conducted, have been found to contain plasmids. We believe that the difference seen in plasmid caused by the fact that plasmids are not stable and they can easily lose and gain plasmids.

In this study and in previous studies carried out (Goni et. al, 2004; Sabour et. al, 2004), different resistance profiles were observed in isolates in genetically close relationships. This can be explained by the fact that; isolates gain resistance genes from other organisms with conjugation, transformation and transduction mechanisms, from the same or different types of staphylococci or other gram positive bacteria with gene transfer.

Methicillin resistance emerges when the chromosomal gene (mecA) which encodes the differences in target proteins is gained by bacteria. The methicillin resistance in staphylococci is achieved through a modified PBP (PBP2a) that is encoded by mecA gene, which is a chromosomal gene (Arslan, 1995; Bergan and Kocur, 1984; Chambers, 1997). This resistance takes place when the penicillinase (beta-lactamase) enzyme and penicillin beta-lactam ring gets hydrolyzed. The genetic data that causes the generation of this enzyme is carried on plasmids, and this facilitates the spread of resistance among staphylococci (Conterno et al., 1998; Coombs, 2002; Ellis et al., 2004).

The reveal of mecA gene via PCR method for the methicillin resistance of staphylococci strains gives way to accurate, sensitive and immediate detection of results. (Acar, 1996; Baron et al., 1994; Sanford et al., 1994; Şahin,2003).

In his research, Uğur Demir Pekin (2006) found mecA positive in all 7 strains (Demirpek, 2006).

In our study, we have studied the presence of mecA. For methiciliin resistance, we have examined the presence of mecA gene in resistant isolates via PCR method, and we have found mecA positive in 49 of the 51 isolates. In the remaining 2 isolates were mecA negative.

We think that resistance in mecA negative samples to result from overproduction of beta-lactamase (borderline oxacilline resistant *S. aureus)* or consist of point mutaion at PBPs. But for confirm this first we should detect methicillin resistance again. In previous study was declerated that oxacillin MIC is 2-8 mg/L at mecA negative samples are BORSA (Song et al., 1987). An other study, compared disc diffusion and oxacillin agar dilution tests with PCR method. They determined that samples, which were determined mecA negative with PCR method, were MRSA with oxacillin agar dilution test. And also they emphasize that these isolates developed resistance with overpruduction beta-lactamese (Eroğlu et al., 2001). Resistance may develop to results of a structural change in PBPs at mecA negative methicillin resistant is explained (Prasad et al., 2000).

CHAPTER 5

CONCLUSION

The nosocomial epidemics caused by methicillin resistant *S.aureus* (MRSA) strains have become a serious health problem all around the world due to the cost of infection control measures and the antibiotics used in treatment (Gorwitz et al., 2006; Goto et al., 1999; Hollyoak and Cunn, 1995).

Infections caused by MRSA is serious health problem as all around the world, its also become a serious health problem in Turkey. (Demirpek, 2006). Related study about resistance in our country shows that, increasing the frequency of antimicrobial resitance in staphylococci and emerged various enzymes that caused resistance (Leblebecioğlu and Ünal, 2002). In various studies, methicillin resistance detected in *S.*aureus. Methicillin resistance found %21 in Ankara, %33 in Istanbul, %26 in Konya (Kocagüz et al., 1997; Arman, 2001; Öztürk et al., 1996; Fındık et al., 1996).

The purpose of this study is to make molecular typing of 51 *S.aureus* which were identified as a cause of hospital infection from Istanbul Cerrahpasa Medicine Faculty. For this purpose in molecular typing of 51 *S.aureus* isolates, we used PCR method which can identified mecA gene quickly and reliably technique and Plasmid profile analysis which is an easily executed and interpreted technique.

In conclusion, we investigated the presence of mecA gene with PCR technique and differances of plasmid patterns with PPA technique. At Staphylococcus to detect the methicillin resistance is very important to early and accurate identification with PCR

technique for treat infections in a short time, unneccesary use of expensive and harmful drug and to be taken in a time to infection control measures.

In this study, in addition to the obtained data, we can be used PFGE technique to discriminate the pulsotypes and made clarification to molecular epidemiology of *S. aureus* isolates by making sequence analysis to pulsotype. Beside this, in infection control could be utilized with molecular data which were obtained in this study and data will be obtained from other studies in other parts of our country.

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